DEC 26 1919

VOL. XL DECEMBER, 1919 No. 2

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

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PUBLISHED MONTHLY
AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
FOR THE JOURNAL OF BIOLOGICAL CHEMISTRY, INC.

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Entered as second-class matter August 1, 1911, at the Post Office at Baltimore, Md., under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorized on June 29, 1918.

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THE EFFECTS OF MALT AND MALT EXTRACTS ON SCURVY AND THE ALKALINE RESERVE OF THE BLOOD.

By J. F. McCLENDON, W. C. C. COLE, O. ENGSTRAND, AND J. E. MIDDLEKAUFF.

(*From the Physiological Laboratory of the University of Minnesota Medical School, Minneapolis.*)

(Received for publication, August 25, 1919.)

It has been well established that scurvy may be prevented or cured by eating fresh raw food. The object of the present paper is to determine whether, in the entire absence of fresh foods, scurvy may be prevented or cured by malt products suitable for food for infants and adults. Cereal grains may be easily transported and stored so as to preserve their sprouting capacity and there would be no necessity of a shortage of them, and hence no necessity of scurvy if the scurvy-ridden communities knew how to use them. Wiltshire has shown that human scurvy may be cured with sprouted beans, and Greig advocates their use in the army, but one of us has tested the sprouting of beans in army camps and under various climatic conditions and found them far less resistant to mould than are any of the cereal grains except maize. Germination tests on many samples of beans bought from civilians showed that they were incapable of sprouting under any conditions. Army beans that showed over 90 per cent germination were attacked by mould if the temperature rose above 15° at night.

The relative importance of this work depends on the prevalence of scurvy, but we have not been able to obtain complete information on this subject. During the Civil War 30,741 cases of scurvy among white troops were recorded. Bruntz and Spillmann designate "trench-foot" as a prescorbutic condition. Many cases of scurvy have been diagnosed as such only after treatment on the supposition that the lesions were due to another cause.

Durand finds that canned milk (which has been repeatedly shown to be deficient in antiscorbutics) causes dental caries in

infants. In examinations of young children whose infant histories were known, 53 to 74 per cent showed dental caries following a diet of canned milk, whereas breast-fed children showed 28 to 42 per cent dental caries. If the soundness of teeth depends on antiscorbutics in the diet, the subject is one of greater importance than it has generally been considered.

Soon after Holst and Fröhlich in Norway began their work on scurvy in guinea pigs, Fürst, working in their laboratory, showed that fresh sprouted barley is sufficient in antiscorbutic principles, but that these properties are lost in the drying of the malt, as is usually done in the beer industry. Weill and Mouriquand have disputed the fact that sprouting barley contains antiscorbutic substances but they admit that the young barley plant is effective in this way. Chick and Hume, and Cohen and Mendel have also found antiscorbutics in sprouting grains (oats, barley).

Since barley grains yield an acid ash and Wright has claimed that scurvy is the result of acidosis, we eliminated this possible objection by determinations of the alkaline reserve of the blood in scurvy as compared with that in health. We used guinea pigs because they are the classical animals for the study of scurvy and also rabbits since they are particularly susceptible to acidosis. In order to control the ash of the food we took two equal weights of barley, sprouted one part, and fed it to one animal, and fed the other part, unsprouted, to an animal of the same size. Brown has shown that the barley grain loses or gains no salts by steeping as long as it is alive and that the salts gained by the steep water come from the husk. Since guinea pigs do not eat the husk, we assume that the one eating dry barley gets the same salts as the one eating the same weight of sprouted barley. The guinea pigs were kept in cages of $\frac{1}{4}$ inch wire screen, with partitions so that each guinea pig had a separate compartment. They learned to drink out of inverted tubes filled with water.

The barley was sprouted by the drum method, large, wide-mouthed glass jars serving as drums. The jars were placed on two parallel steel shafts, 2 cm. in diameter, 200 cm. long, and placed 10 cm. apart. Both shafts were rotated in the same direction by means of a small electric motor and worm-gear. The friction of the shafts on the jars caused them to rotate very slowly. Cool, moist air was supplied by means of an aspirator pump and trans-

mitted by tubes projecting into the mouths of the jars. Two adjacent jars were placed with their mouths facing one another and air was supplied to both of them by means of a T-tube. Seed barley[1] was placed in a jar together with the amount of water it would absorb in 24 hours rotation, and at the end of that time it was washed, drained, and the rotation continued until the sprouting was complete. If the temperature within the jar approached 30° during the first 48 hours, death of some grains might occur, but after the first 48 hours (or, more correctly, after the

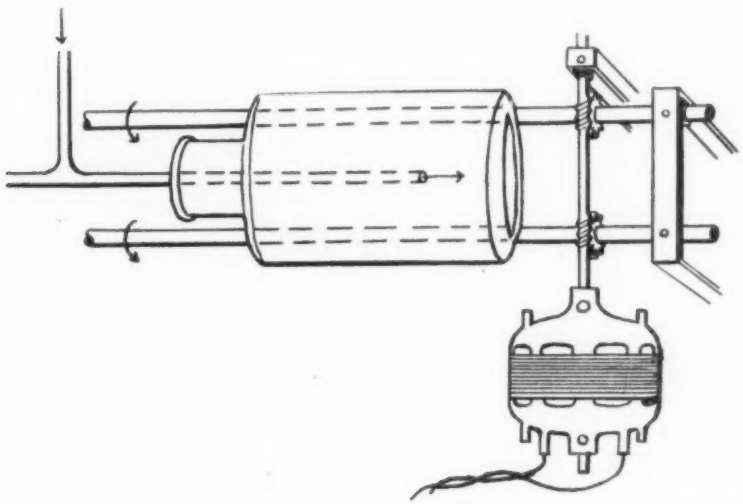

FIG. 1.

acrospire appeared), the temperature might be raised to 30° with impunity. Since rise in temperature decreases the time required for sprouting, we never let it fall below 20°. The original design of the apparatus is shown in Fig. 1.

In determining the alkaline reserve, twelve guinea pigs and six rabbits were used. The animals were weighed every day, but the weight curves in this paper are smoothed so as to obliterate the confusing details since we were unable to compare the curves

[1] We are indebted to the cooperation of Professor A. C. Arny for the seed barley.

in their original form. The weight curves of the guinea pigs are shown in Fig. 2. The spaces on the ordinate represent differences of 100 gm. in weight, and the spaces on the abscissa intervals of 30 days. The curves of the animals fed on sprouted barley (with acrospire 1 inch long) are represented by unbroken lines, and the curves of the animals on dry barley by broken lines. The animals are numbered 1 to 6 beginning with the smallest (200 gm.) at the left. It may be seen that the animals on sprouted grain lost little if anything in weight, whereas those on dry grain lost rapidly.

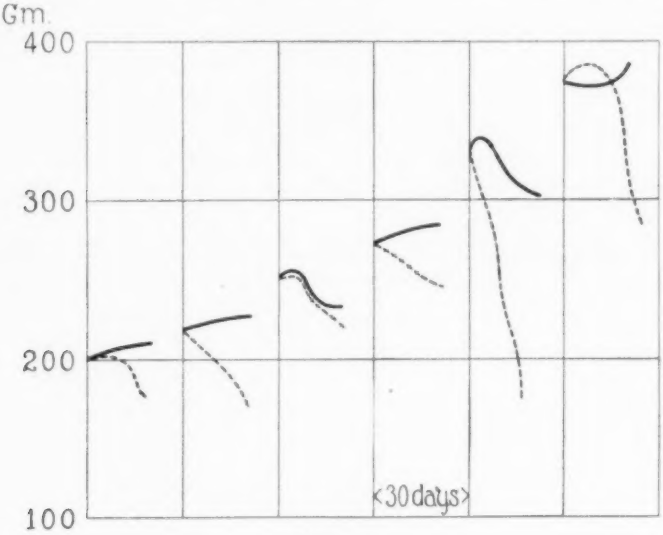

FIG. 2.

This was not due to lack of water as both sets were supplied with water *ad libitum*, and the dry grain was fed in a dish of water. The fifth guinea pig on dry grain died in 17 days, the remaining were bled to death in 20 to 25 days, and all showed hemorrhages characteristic of scurvy. None of the guinea pigs on sprouted grain showed scurvy symptoms.

The alkaline reserve was determined by a modification of the Van Slyke, Stillman, and Cullen method. We found their method satisfactory, but modified it a little so as to compare more readily our previous data with the present determinations.

Neutral red fades in the standard solutions and we found that dibromothymolsulfonephthalein was more permanent and gave striking color changes, but we did not have enough of it and confined our determinations to neutral red, making new standards every day. For standards, we used the borax mixtures of Palitzsch since they are better and cheaper than phosphate mixtures. Since Palitzsch does not show the continuous curve for these mixtures, and we determined ours with the hydrogen electrode, we give the following directions for making them.

We made stock solutions of 0.05 molecular borax and 0.2 molecular boric acid and protected them in resistance glass flasks with automatic burettes with soda-lime tubes. We used reagent borax dried in air as it came from the manufacturer, noting that none of the crystals was moist or had effloresced. We recrystallized the boric acid and dried it in a desiccator. The water was freshly distilled and CO_2-free air was blown through it for 15 minutes. The following table shows the pH and the per cent of the borax stock solution, the remainder being boric acid stock solution.[2]

pH.....	6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	7.9
Per cent of borax.	2.5	2.7	3.2	3.9	5.0	6.1	7.4	9.9	10.6	12.7	15.1	17.7	20.5	23.7
pH.....	8.0	8.1	8.2	8.3	8.4	8.5	8.6	8.7	8.8	8.9	9.0	9.1	9.2	
Per cent of borax.	27.3	30.9	35.2	39.7	44.4	49.3	54.7	60.7	67.4	74.5	81.5	89.2	96.8	

We found that the dilution of plasma recommended by Van Slyke is sufficient, as we got practically the same results with double or quadruple that dilution, but that the CO_2 remaining in the plasma after rotation made a difference of about two drops in the titration. This is no objection to Van Slyke's method, but we wished to blow out the CO_2 as thoroughly as we did in the electrometric method in order to compare results by the two methods. The flask was rotated by an electric motor while a stream of CO_2-free air was blown through it, as shown in Fig. 3.

[2] We are indebted to Professor Grace Medes for most of the work in preparing the standards.

The method may be summarized as follows: Tubes were prepared for the blood by drying in them enough 25 per cent potassium oxalate solution to make 0.2 per cent dry oxalate in the blood. The animal was anesthetized lightly with ether and the blood was drawn from the left ventricle (carotid in rabbits) and centrifuged immediately. 1 cc. of plasma was introduced in a 100 cc. flask of resistance glass and 20 cc. of distilled water, 0.3 cc. of 0.1 per cent solution of neutral red, and 3 cc. of 0.01 N HCl were added. Three similar flasks were made up with 25 cc. of

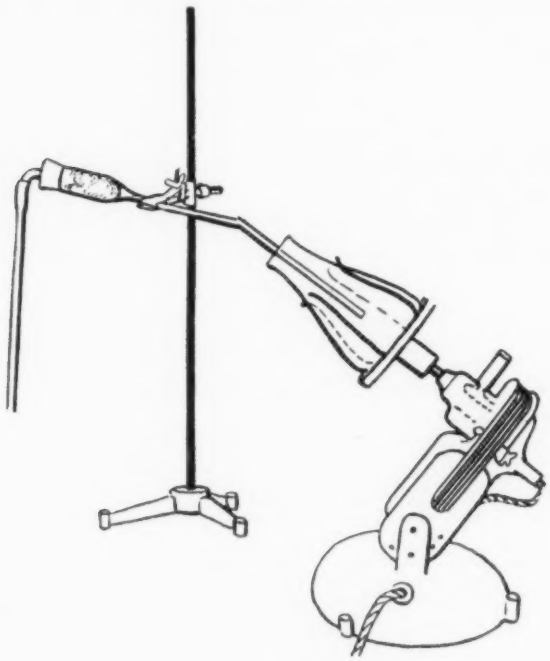

FIG. 3.

standard solution and 0.3 cc. of indicator in each, showing pH = 6.6, 7.0, and 7.4. The plasma flask was rotated 5 minutes and, if the color came within range of the standards, more acid was added, but it is not necessary normally to add more acid. The plasma was then titrated in the same flask with 0.01 normal solution of CO_2-free NaOH to the three standard colors in succession. Both

acid and alkali were made up with CO_2-free distilled water and kept in automatic burettes that fill from the top so that the solutions do not pass through a greased stop-cock before entering the burette.

The following table shows the alkaline reserve in terms of a normal solution, as titrated to the three standards. Owing to changes in ionization by dilution, we prefer not to say what pH is the correct end-point, but any end-point may be noted by drawing a curve through the three points.

No.	Scurvy guinea pigs (dry barley).			Controls (barley with 1 in. sprouts).		
	pH			pH		
	6.6	7.0	7.4	6.6	7.0	7.4
1	0.026	0.024	0.021	0.016	0.014	0.012
2	0.021	0.019	0.017	0.018	0.016	0.014
3	0.034	0.032	0.026	0.021	0.019	0.017
4	0.020	0.019	0.017	0.020	0.019	0.017
5				0.022	0.021	0.019
6	0.022	0.020	0.017	0.019	0.018	0.016

It may be seen from the table that with the exception of Scurvy Guinea Pig 3, which was in a moribund condition with dark venous blood, there is no essential difference between the alkaline reserve of those with and without scurvy. If the alkaline reserve changed during ether anesthesia, and differently in different individuals, that would account for the slight differences shown, but not for the similarities of the two groups. We must conclude that acidosis has nothing to do with scurvy in the guinea pig.

The weight curves of the rabbits are shown in Fig. 4, those on sprouted grain are shown by unbroken lines and those on dry grain by broken lines. The spaces on the ordinate represent differences of 300 gm. in weight, and the space on the abscissa represents an interval of 20 days. The rabbits were numbered 1 to 3 beginning with the pair at the top (900 gm.). The rabbits on sprouted barley remained nearly constant in weight, while those on dry barley lost and two of them died before blood was drawn. The 900 gm. rabbit on dry grain showed scurvy lesions when blood was drawn on the 21st day. Before the beginning of the experiment, all the rabbits had been kept together and fed on oats and

hay. Hence they were particularly susceptible to a scorbutic diet. The alkaline reserve is shown in the following table.

No.	Scurvy rabbits (dry barley).			Controls (barley with 1 in. sprouts).		
	pH			pH		
	6.6	7.0	7.4	6.6	7.0	7.4
1	0.008	0.006	0.003	0.009	0.008	0.004
2				0.011	0.010	0.008
3				0.007	0.006	0.004

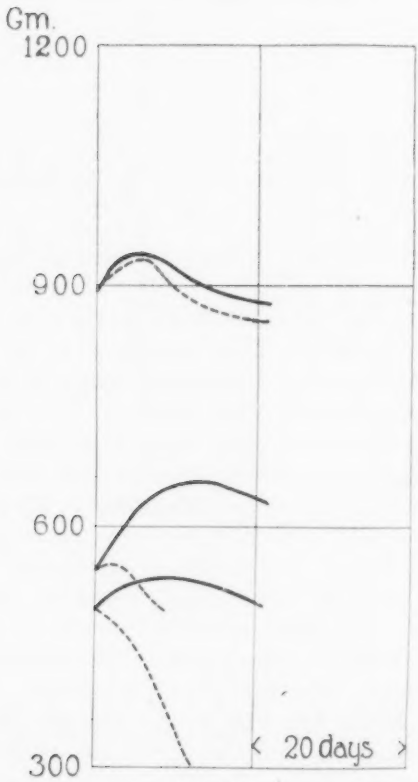

FIG. 4.

In our previous determinations on rabbits fed on carrots and hay (McClendon, von Meysenbug, Engstrand, and King), the

alkaline reserve was found to be about 0.020, and hence both sets of the above rabbits show acidosis due to the deficiency of alkali in the exclusive barley diet. The scurvy rabbit does not show a significant difference in alkaline reserve from the controls, and therefore we may conclude that acidosis has nothing to do with scurvy in rabbits, but may be a factor in the early death of the animals.

According to Fürst, barley sprouted for 3 days prevents scurvy, whereas Weill and Mouriquand found that 10 days sprouting was required. These workers do not record the temperature, and since the rate of sprouting is more than doubled by a rise of 10° in temperature, it is necessary to control and record the temperature or determine the degree of sprouting by the length of root and acrospire. In the following experiment an attempt is made to determine the degree of sprouting and quantity of malt necessary to prevent scurvy in the guinea pig, but an epidemic of pneumonia made the data fragmentary.

In this experiment barley seedlings of three ages were used (1 day, 2 days, and 3 days). In 1 day the roots were $\frac{1}{8}$ inch in length, in 2 days the acrospire was visible, and in 3 days the acrospire projected $\frac{1}{2}$ inch beyond the grain. The growth curves of the guinea pigs are shown in Fig. 5. Fifteen of these guinea pigs were divided into five sets, numbered 1 to 5, and arranged from left to right (Fig. 5). The curves of the guinea pigs fed on 1 day sprouts are shown by unbroken lines, those on 2 day sprouts by broken lines, and those on 3 day sprouts by dotted lines. The divisions on the ordinate represent differences of 100 gm. in weight, and those on the abscissa intervals of 30 days. The first set was fed 1 gm. of sprouts, the second set 2 gm., the third set 3 gm., the fourth set 4 gm., and the fifth set 5 gm. per 100 gm. of body weight of guinea pig per day, and they had oats and water *ad libitum*.

It may be seen from the curves that nearly all the guinea pigs began to gain in weight and then to lose; the end of the curve represents the early death. Besides this series, one guinea pig, represented by the chain of circles in Fig. 5, was fed oats and 2 day sprouted barley *ad libitum* and he ate less than 25 gm. per day of the latter. He died at the end of 33 days with no symptoms of scurvy. The fact that guinea pigs of this weight die of scurvy

in about 20 days indicates that 2 day sprouts contain an appreciable amount of antiscorbutic substance. Another guinea pig, represented by the chain of crosses in Fig. 5, was fed oats and 3 day sprouted barley *ad libitum*, and ate less than 25 gm. per day of the latter. He gained in weight for 32 days. The decline in weight following was apparently accompanied by an infection that caused death of some of the guinea pigs, but was manifested by a coryza and dyspnea in this one. At the end of the 48th day we discontinued the oats and gave him the sprouts and autoclaved

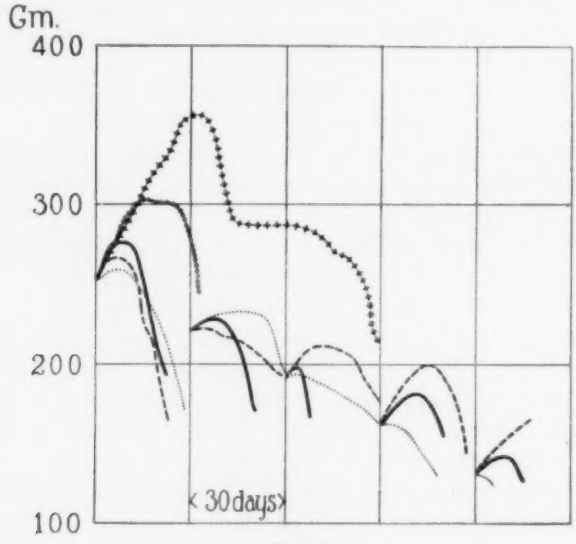

FIG. 5.

biscuit containing condensed milk for a few days, and then fed him exclusively on sprouted barley and water until he died at the end of 90 days without symptoms of scurvy. Since barley is deficient in salts, protein, and fat-soluble A, as shown by Steenbock, Kent, and Gross, and salts, at least, cannot be synthesized in the sprouting of the barley, the death of this guinea pig might have been due to lack of salts.

Since the above experiments show that barley, sprouted until the acrospire is $\frac{1}{2}$ inch long or longer, contains considerable antiscorbutic substance (and the same is true of other grains) efforts

were made to prepare sprouted grain for human food and yet preserve the antiscorbutic substance. The husks of barley make it poor eating, but wheat and rye offer no mechanical difficulties. If sprouted wheat and rye are heated to 70°, the starch is gelatinized and it may be eaten as a salad or breakfast food. Three guinea pigs were fed exclusively with wheat and rye sprouted until the acrospire projected ½ inch beyond the grain and placed in water of 70° until the starch was gelatinized. Their growth curves are the first three beginning at the left in Fig. 6. The weight is marked on the ordinate and the divisions on the abscissa represent intervals of 30 days. None of these animals showed symptoms of scurvy, but two of them died rather early. The

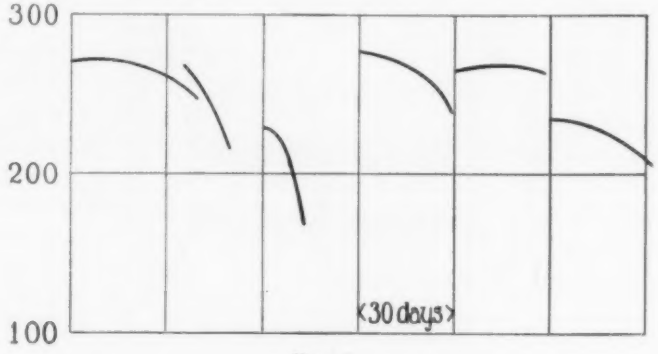

FIG. 6.

fact that one of them lived 34 days and showed no scurvy symptoms at autopsy shows that the sprouted wheat and rye contain antiscorbutic substances which are not destroyed by heating to 70° to gelatinize the starch. Another guinea pig shown in Fig. 8 was cured of scurvy by a similar diet.

Since the whole grain cannot be fed to infants, we attempted to prepare a juice containing the antiscorbutic substance. Since the antiscorbutic substance probably exists in the cells of the acrospire or roots, and it is difficult to crush these cells, we used a special mill for the purpose, shown in Fig. 7. This mill has polished steel rollers, 2.25 inches in diameter, and differs from an ordinary malt mill in that the rollers are geared to one another and there are scrapers to remove the crushed sprouts. A pulley wheel, 2 feet in diameter, was fitted to one of the rolls and driven

by a belt. Sprouted wheat or rye showed a tendency to slide out of the groove between the rolls, but sprouted barley fed well into the mill owing to the roughness of the husk. After the sprouted barley was crushed between the rolls it was placed in a canvas bag in a press capable of exerting a pressure of 5,000 pounds to the square inch. It was found, however, that very little juice came out of it at full pressure and a new bag had to be used each time. By adding water before pressing, less pressure was sufficient and the bag lasted longer.

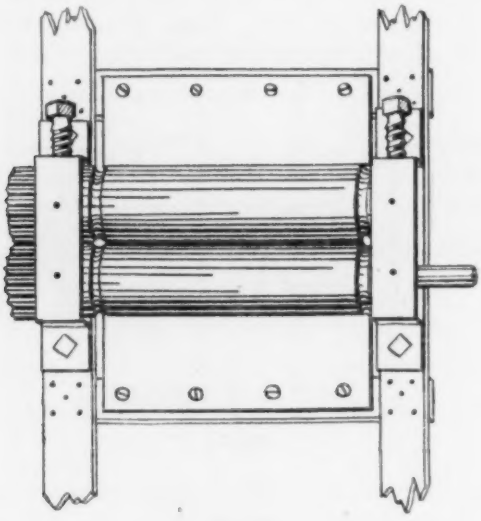

FIG. 7.

Three guinea pigs, whose weight curves are shown in the right half of Fig. 6, were fed with the juice of barley with the acrospire ½ inch beyond the grain and water *ad libitum*. They almost maintained their weight, lived about 30 days, and showed no signs of scurvy at autopsy. One guinea pig, shown in Fig. 8, was cured of scurvy with this juice. This indicates that the juice contains sufficient antiscorbutic substance. It has a grassy taste, but probably could be fed to babies without much difficulty. No doubt the grain could be practically freed from bacteria before sprouting (Duggar and Davis).

Since very little starch is hydrolyzed during the sprouting and most of it remains in the press, an attempt was made to mash the green malt so that the carbohydrate would appear in the extract. Barley with sprouts ½ inch long (acrospire extending ½ inch beyond the grain) was crushed by passing it through a clothes wringer with rubber rolls, mixed with twice its weight of water, and heated to 70° to gelatinize the starch. It was allowed to remain in the same vessel until the starch iodide reaction disappeared, which required about an hour, and then strained and pressed so that the extract ran into a glass distilling flask with a side neck. A rubber stopper was inserted through which passed a capillary tube extending to the bottom of the flask. The flask was placed in a water bath, heated to 70°, and suction was applied to the side neck so as to evaporate the contents. A fine stream of air bubbles, liberated from the end of the capillary tube, prevented bumping. When the malt extract was evaporated to the consistency of a very thick syrup, *i.e.* the thickest syrup that could easily be removed from the flask, it was stored in glass jars until used. A slight amount of oxidase remained in the extract and caused a very slow darkening of the surface exposed to air. Fermentation was prevented by the evaporation, but mould would grow very slowly on the surface if planted there. Some of this extract has been kept for 5 months in good condition.

The weight curves of the guinea pigs fed on this malt extract are shown in Fig. 8. The body weight is marked on the ordinate, and the divisions on the abscissa represent intervals of 25 days. The guinea pigs were divided into six pairs which were fed on increasing quantities of extract from left to right, 0, 5, 10, 15, 20, and 25 gm. per guinea pig per day mixed with a dough or mush. The mush for one guinea pig consisted of 12 gm. of Graham flour, 12 gm. of rolled oats, 12 cc. of evaporated milk, and 1 cc. of a salt solution containing 25 per cent NaCl and 6 per cent $CaCl_2$, together with the designated quantity of extract. The mixture was a mush only with the larger quantity of extract, and a stiff dough with the smaller quantities. Water was given *ad libitum* as in all the above experiments. All the animals lost weight finally, although they ate greedily until they all showed symptoms of scurvy (tender swollen joints, falling hair, and loose molars) at about the 15th day, after which they ate less. The larger guinea pig of the fourth

pair showed marked scurvy on the 16th day and was transferred to a diet of sprouted wheat and rye heated to 70°, as shown by the broken line continuation of his curve in Fig. 8. He gained considerably in weight, and the scurvy symptoms rapidly disappeared so that none was found at the autopsy on the 26th day. This indicates that the process of heating to 70° to gelatinize the starch does not destroy the antiscorbutic substance. The reason all the

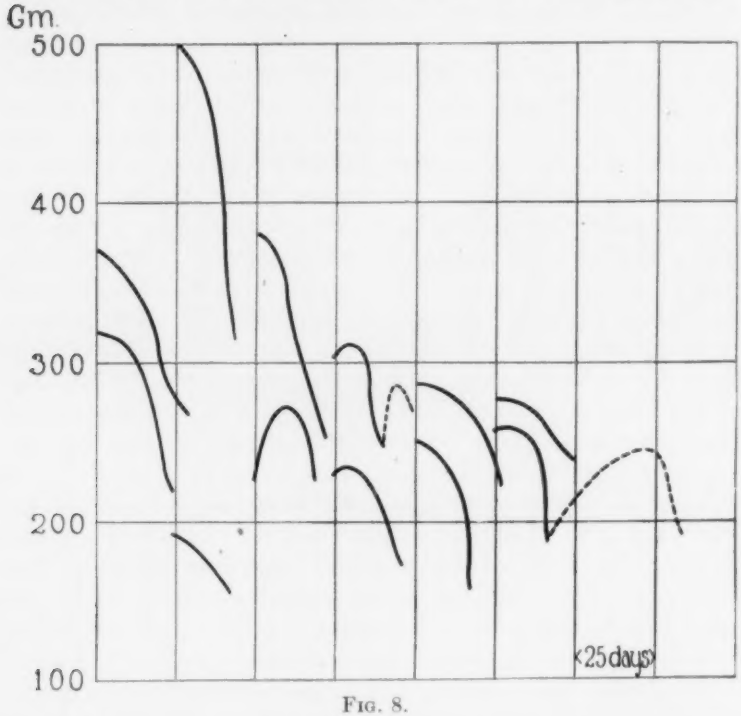

FIG. 8.

animals developed scurvy may lie in the possibility that the sprouts were not sufficiently crushed and the antiscorbutic substance was never extracted from them. The smaller guinea pig of the sixth pair developed marked scurvy with swollen wrists (which when touched provoked a squeal), loose molars, and bloody diarrhea. About the 20th day he could scarcely move about his cage and was changed to a diet of raw juice of sprouted barley crushed between steel rolls, as shown by the broken line continuing

his curve in Fig. 8. He gained rapidly in weight and lost all scurvy symptoms but finally began to lose and died on the 60th day showing no signs of scurvy on autopsy.

With the exception of the two animals cured of scurvy, the diagnosis of scurvy in all of the twelve was confirmed on autopsy. The first pair, receiving no malt extract, lost weight but little more rapidly than the average of those fed extract. Since Fürst has shown that commercial malt extract is deficient in antiscorbutic substance, and the same is true of beer according to Smith, it seems probable that the only way to get the antiscorbutic substance into the extract is to crush the green malt between rolls that thoroughly break up the cells of the acrospire. It is also desirable to sprout the grain to a more advanced stage than is done merely for the development of diastase.

At autopsy some guinea pigs showed impacted cecums, but this was only in case the animal died of scurvy and never if it was killed when the scurvy symptoms first appeared. We assume that the impacted cecum, as observed by McCollum and Pitz, is due to the fact that the guinea pig drinks little water during the last day or so of its life.[3]

CONCLUSIONS.

Acidosis has nothing to do with scurvy.

Sprouted cereal grains, especially after the acrospire projects ½ inch beyond the grain, are rich in antiscorbutic substance (in this we merely extend the work of Fürst, and Cohen and Mendel).

The antiscorbutic substance in sprouted grain is not destroyed by heating to 70° to gelatinize the starch.

The antiscorbutic substance may be extracted from sprouted barley after crushing it between steel rolls that are so close together that the cells of the acrospire are crushed. In order to make the green malt feed between the rolls they must be geared to one another so as to turn at the same rate.

[3] The keeper of the stock rabbits said he fed one rabbit in a separate cage exclusively on oats for 9 months. One of us at autopsy of this rabbit found no marked gross lesions except a fragility of the bones. One rib had broken spontaneously. Rabbits are much more resistant to scurvy than are guinea pigs.

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TOXICITY OF PHENYLACETIC ACID.

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(Received for publication, October 7, 1919.)

The retention of protein material in the intestine and its subsequent putrefaction leads to the formation and absorption of many protein decomposition products which are more or less harmful to the organism.

Phenylalanine on putrefaction yields three different aromatic acids; phenylpropionic acid, phenylacetic acid, and benzoic acid, while tyrosine undergoes analogous decomposition.

However, if phenylpropionic acid (1) is introduced into the gastrointestinal tract and absorbed, no phenylacetic acid is formed but it is subjected to the process of β-oxidation, loses two C atoms, and is changed directly into benzoic acid.

The benzoic acid combines with glycocoll and is excreted in the urine as hippuric acid. *p*-Hydroxyphenyl propionic acid (1) in a like manner is oxidized to *p*-hydroxybenzoic acid and is excreted as *p*-hydroxyhippuric acid. Phenylacetic acid resists oxidation and remains to be altered by combination with other compounds.

Salkowski (2) considered the acid relatively non-toxic and believed that it existed even in normal human urine as the free acid. This was perhaps because he had found only the combined acid in the urine of animals, such as dogs (2, 3), rabbits, and horses and was unable to isolate either the free or combined acid from human urine.

Huppert (4) fed phenylacetic acid to patients suffering from alkaptonuria and proved that the acid aided no way in the formation of homogentisic acid but was unable to find even a trace of the acid after feeding the patient a 10 gm. dose.

Hotter (5), who ingested the acid himself, could find neither the combined or uncombined acid in his urine, so concluded

that it was oxidized to benzoic acid and excreted as hippuric acid. Phenylacetic acid is particularly interesting from a physiological standpoint on account of its different metabolic action in the organism of man, animal, and fowl.

In the human body, the acid is combined with glutamine and excreted as phenylacetyl glutamine (6). This is the only case so far recorded where the amino-acid glutamine has been used by the body for the purpose of detoxicating a poisonous substance. Animals fed on phenylacetic acid detoxicate it by joining it with glycocoll and excreting it as phenaceturic acid (2).

After feeding the acid to a hen, Totani (7) isolated a compound from the excreta which he terms phenacetornithuric acid. This substance is a combination of one molecule of ornithine with two molecules of phenylacetic acid.

Phenylacetic acid, while found in only small amounts in the normal human body is one of the most important protein putrefaction products and is by no means as non-toxic as was previously supposed.

A hen weighing 2.23 kilos, after receiving 1 gm. of the acid, refused to eat. A second dose of 1 gm. 3 days later caused the hen to lose weight and develop marked signs of intoxication. A dog weighing 32.6 kilos was able to take 3 gm. of the acid with no apparent signs of discomfort; however, after receiving a dose of 7 gm. of the acid it became very thirsty, refused to eat, seemed to be greatly nauseated, and vomited several times.

A monkey of 4.2 kilos body weight, which received a dose of 1 gm. and refused to eat for several days, also developed a marked diarrhea. Twelve adult humans (male) after ingesting 5 gm. each of the acid showed in every case practically the same symptoms. The sodium salt of the acid was dissolved in 200 to 300 cc. of water and rapidly drunk. In 15 to 30 minutes after the ingestion of the acid, the subject became thirsty and this symptom was rapidly followed by a feeling of hunger. If food was ingested, symptoms of nausea developed; in case no food was taken, a feeling of dizziness resulted, followed either by drowsiness or increased nervousness. One subject weighing 59.1 kilos ingested as much as 16 gm. of the acid within a period of 2 hours. Within a few minutes, the usual sensation of dizziness and hunger developed, so the subject partook of an unusually heavy meal and went to bed.

1 hour after ingesting the last of the acid, he was unable even to stand unsupported. After sleeping soundly for 6 hours, he awoke and drank 1 liter of water, immediately fell asleep, and again slept soundly for nearly 8 hours. On waking he demanded water and drank more than 1½ liters. He complained of nausea, headache, pain in the eyes, and of loud ringing in the ears. He was able to sit alone but seemed unable to stand unsupported or to correlate his movements. After another 4 hours of sleep, he appeared quite refreshed and normal in every way. In this case there was no sign of diarrhea but on the contrary he presented an obstinate case of constipation, which lasted for about 3 weeks.

In many respects the symptoms of poisoning by this acid resemble those of alcoholic poisoning.

EXPERIMENTAL.

In order to determine the toxicity of the acid, we decided to feed a small dog increasing doses of the substance and to determine if possible the minimum dose which would cause death and to study as carefully as possible any pathological changes produced by the acid.

A small dog of 7.5 kilos body weight was selected and placed in a metabolism cage for observation. The acid was fed to the dog as a water solution of the sodium salt by means of a stomach tube. On the 1st day of the experiment, he received 1 gm. of the acid. On each succeeding day, the dose of the acid was increased by 1 gm.

During the first 24 hours of the experiment following the 1 gm. dose of the acid, the dog showed no signs of discomfort but ate as usual and showed no signs of abnormal thirst. On the 2nd day after receiving the 2 gm. of the acid, the dog showed an abnormal appetite and drank often but only a small amount of water each time. On the 3rd day, he showed signs of drowsiness, but ate as much as usual and drank a large amount of water. On the 4th day, the dog refused to eat, spent most of the time in sleep, and seemed scarcely able to stand when removed from the cage. He was unable to walk and weighed at this time only 6.35 kilos. On the 6th day he still refused to eat and seemed to be in a semicomatose condition.

Up to this time there had been no signs of albumin in the urine but at this point a sufficient quantity was present to give a decided reaction. On the 7th day of the experiment, the dog appeared very weak and after receiving 7 gm. of the acid, underwent a series of convulsions during which time he vomited most of the acid. As much of this acid as possible was reclaimed and weighed. The total amount vomited was approximately 5.5 gm. so the dog received in fact only about 1.5 gm. of the phenylacetic acid on the 7th day.

For about 2½ hours after receiving this last dose of the acid, he appeared quite lifeless, then suddenly underwent a second series of convulsions, which ended in death.

Autopsy.—Performed about 6 hours after death. Male dog weighing 6.30 kilos. Gross examination of the organs presented no morphological lesions with the exception that the kidneys on section were congested and somewhat swollen, the cortex being pale; medulla congested, capsule non-adherent. Portions of liver, kidney, spleen, stomach, and alimentary canal were taken for microscopic examination. The specimens were fixed in Orth's fluid and mounted in paraffin. Sections were cut 6 microns in thickness and examined with $\frac{1}{12}$ oil immersion, ocular 10.

Microscopic Examination.—The tunica fibrosa of the kidney does not appear to be thickened and the nuclear elements show no deficiency in staining qualities. The capillaries in the cortex corticis are engorged and the cellular elements within them appear disintegrated. While there is a general engorgement of the blood vessels, of the cortical portion of the organ, there is no extravasation of blood in the interstitial tissue.

The epithelium of the proximal convoluted tubules is much swollen and granular, so that the lumen of the tubule is in many places completely occluded by the approximation of the distal edge of the epithelial cells. Some of the tubules contain in their lumen the remains of broken down epithelial cells but this is in localities distinct from those in which the lumen is occluded and may indicate epithelial areas, which bore the effects of a greater toxic action of the drug. Blood elements are not seen within the tubules.

A glistening, hyaline material is found in the lumen of many of the tubules and in the cytoplasm of many cells the same material is seen. The engorgement of the capillaries between the tubules is marked. The degeneration of the epithelium is most marked in the proximal convoluted tubules in the neighborhood of the Malphigian corpuscles, becoming less marked as the descending loop is approached.

The arched collecting tubules are filled with the hyaline material and their epithelium, including the nuclei, is in many places destroyed. The epithelium of Bowman's capsule is likewise destroyed in many of the

renal corpuscles and while shrinkage of the glomerulus from the capsule is not present in every instance, yet it occurs in many of the corpuscles, particularly in those near the boundary zone of the medulla, and in the capsular space an exudate of hyaline and granular material and blood cells is present.

A round cell infiltration of the stroma of the glomerulus occurs and the capillaries of the tuft are engorged with blood.

In the medulla of the kidney, both limbs of Henle's loop show marked destructive changes of their epithelium. This in many places is totally disintegrated, so that the lumen of the tubules is filled with a mass of cellular remains and misplaced nuclei and a fine reticular mass, staining deeply with eosin, is present. Such epithelial cells as are not destroyed are detached in places from the wall of the tubules.

The epithelium of the straight collecting tubules does not appear affected by the action of the drug. All the cells are in place, the nuclei distinct, and the cytoplasm is clear. The nuclear membrane is distinct and the lumen of the tubule, for the most part, empty.

The interstitial tissue was not altered in appearance or amount.

The microscopic examination of the liver shows the cells to contain a number of globular refracted spaces, varying in size and in some instances occupying the greater part of the cell. The staining quality of many of the nuclei of the liver cells was markedly deficient. Unfortunately a section was not stained for fat.

The spleen was negative.

The stomach and intestines presented nothing of note.

SUMMARY.

The microscopic findings would seem to indicate that as a result of excessive doses of phenylacetic acid in the dog, the secreting epithelium of the proximal convoluted tube of the kidney is markedly affected; that the endothelium of the blood vessels is not affected; that the epithelium of the arched collecting tubule shows evidence of a destructive action, while that of the straight collecting tubule appears to escape.

The secreting epithelium of the limbs of Henle's loop is most distinctly involved; the fact that the interstitial tissue of the kidney is not injured and that the liver changes is in all probability secondary.

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THE CHEMICAL IDENTIFICATION OF THYROXIN.

SECOND PAPER.*

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(Received for publication, September 15, 1919.)

Thyroxin is a white, highly crystalline substance, odorless, and tasteless. It may be separated from aqueous or alcoholic solutions in microscopic crystals which are not soluble in any organic solvent, except those which are strongly basic or acidic in nature. It is soluble in alcohol in the presence of mineral acid or an alkali metal hydroxide. It is stable toward heat, and its melting point is in the neighborhood of 250°C. Since it is odorless and colorless and is not easily affected by oxidation and reduction, its most important chemical and physical properties are concerned with the acidic and basic groups within the molecule. Thyroxin is a weak acid, but possesses basic properties in the presence of mineral acids.

In 1915, it was suggested that the organic nucleus in thyroxin is indole (1). Its solubility in alkali metal hydroxides, but not in carbonates, indicated that it was of phenolic nature, and its salt-forming power with acids was attributed to an imino group. After it was known that thyroxin contained about 60 per cent of iodine, and before the empirical and structural formulas were determined, the chemical properties of the molecule were best expressed by di-iodo-di-hydroxy-indole.

The first derivative of thyroxin, which helped to give an insight into its chemical structure, was the sulfate. Thyroxin which was precipitated from alkaline alcohol by acetic acid was found to contain 65 per cent of iodine. Thyroxin, precipitated by boiling an aqueous ammoniacal solution, also contained 65 per cent of iodine. Thyroxin, precipitated by adding sulfuric acid to an aqueous alkaline solution and boiling, was found to contain 60 per cent of iodine. The difference in iodine content was shown to be due to the formation of a salt with sulfuric acid, and by estimating the molecular weight of thyroxin from the molecular weight of

*First paper is published in *The Journal of Biological Chemistry*, 1919, xxxix, 125.

sulfuric acid, it was found to be 585. With hydrochloric acid substituted for sulfuric, an iodine content, slightly higher than theoretical, indicated that the hydrochloride was hydrolyzed to some extent. Thyroxin in free form precipitates as needles, but the hydrochloride separates in flat, rectangular, and star-shaped plates. Examination of the crystals of the hydrochloride, which contained more iodine than theoretical, showed both the free form and the

FIG. 1. The hydrochloride of thyroxin which separates in flat plates, rectangular, or star-shaped.

hydrochloride. The sulfate of thyroxin does not hydrolyze with water so readily as the hydrochloride (Figs. 1 and 2).

Ultimate analysis of thyroxin gave the percentages of carbon, hydrogen, oxygen, nitrogen, and iodine, and from these and the molecular weight determination of 586 the empirical formula was shown to be $C_{11}H_{10}O_3NI_3$. In constructing the structural formula we were guided by the following:

1. Acidic Properties.—Thyroxin is readily soluble in sodium, ammonium, and potassium hydroxide, and is insoluble in sodium, ammonium, and potassium carbonate as ordinarily tested. It is soluble in aqueous sodium and potassium carbonate, however, if very little carbonate is added and the solution boiled. It is precipitated by carbon dioxide from an alkaline solution. The empirical formula and these acidic properties, therefore, suggest the presence of one carboxyl group, which has very weak acidic properties, and a hydroxy group.

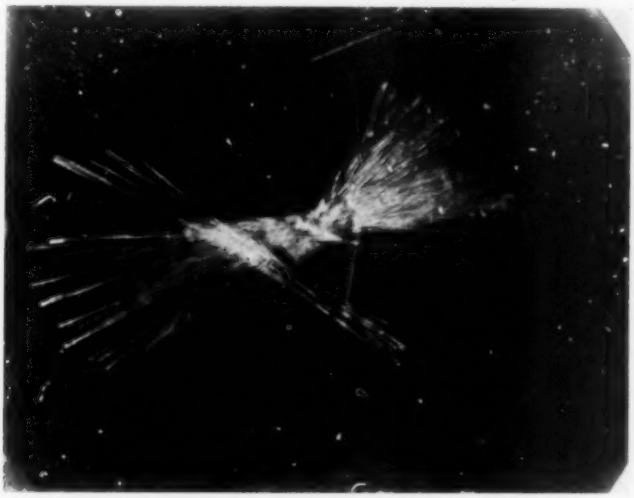
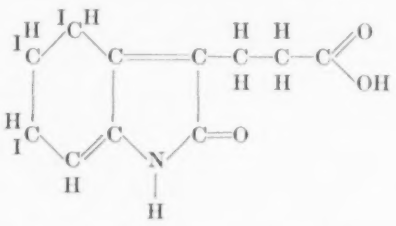


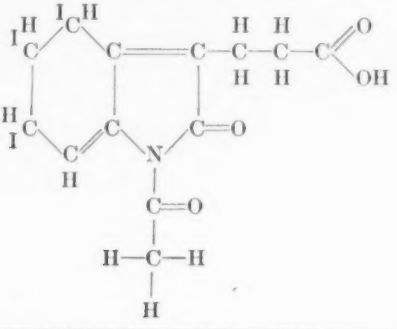
FIG. 2. The free form of thyroxin separated as a sheaf of needles.

2. Basic Properties.—Thyroxin forms salts with mineral acids, but not with weak organic acids. This, together with the fact that thyroxin forms a ureide with cyanic acid, is evidence for the presence of an imino group. The identification of the indole nucleus by the pine-splinter reaction after alkaline fusion was evidence that the imino group was present as in indole. Accepting the presence of the indole nucleus, there remained three extra carbon atoms, a carboxyl group, a hydroxy group, three atoms of iodine, and three extra hydrogen atoms, whose positions in the molecule were to be determined. Since tautomerism is common in the indole group, it seemed probable that the position of the

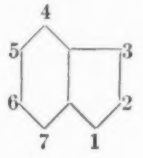
hydroxy group was adjacent to the imino, and that the three carbon atoms including the terminal carboxyl were attached to No. 3 position[1] of the indole nucleus. This structural formula, approximating that of tryptophane, satisfied all that was known concerning the chemical properties of the molecule except the position of the three iodine atoms and the three extra hydrogen atoms. As no special difference was demonstrable between the reactivity of the three atoms of iodine, it seemed most probable that they were all attached to the benzene ring, and as three extra hydrogen atoms would be required, if the iodine was added to, and not substituted for, hydrogen on the ring, they also were placed on the benzene ring. This formula is a tetra-hydro derivative of indole, the three atoms of iodine being substituted for three of hydrogen on the reduced benzene ring.



4, 5, 6 tri-hydro-4, 5, 6 tri-iodo,-2 oxy,-beta indolepropionic acid.



[1] In this paper the positions in the indole nucleus will be referred to as follows:



In proving the formula the first derivatives were those involving the imino group. By the addition of acetic anhydride to a slightly alkaline, alcoholic solution of thyroxin, the hydrogen of the imino is replaced with acetyl and the acetyl derivative may be separated in crystalline form by the addition of sulfuric acid and water and the removal of the alcohol by boiling under

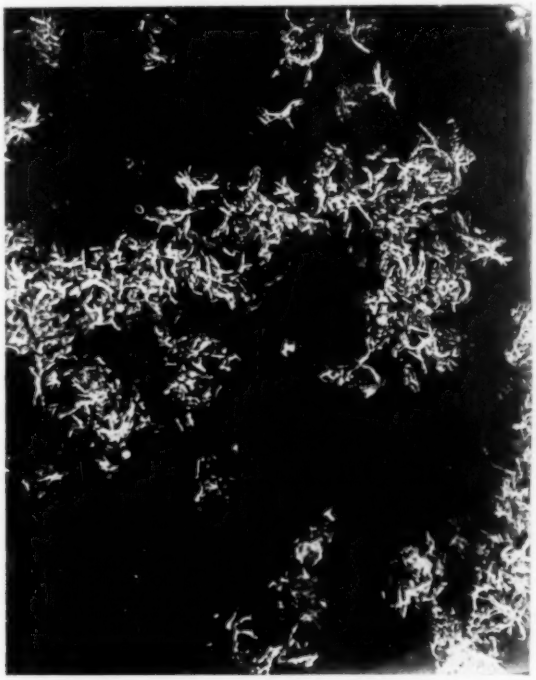
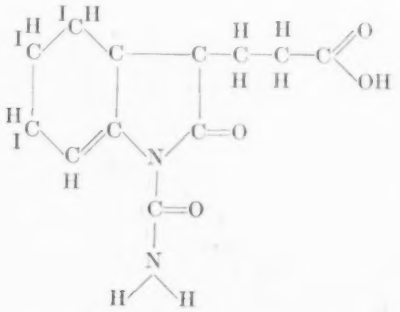


FIG. 3. The crystal form of the acetyl derivative of thyroxin.

diminished pressure. The sulfate of the acetyl of thyroxin is thus formed. This is dissolved in a small amount of alcohol and when added to boiling water the acetyl of thyroxin separates in pure form (Fig. 3). The melting point of the acetyl is slightly lower than that of thyroxin, it crystallizes in the form of needles more curved and much shorter than those of thyroxin, and although thyroxin is insoluble in all organic solvents the acetyl is readily

soluble in alcohol, ether, ethyl-acetate, and dilute aqueous ammonia and pyridine. The close approximation, by analysis, of the theoretical percentage of iodine in the acetyl, 60.77, corroborates the molecular weight of 585.

Another derivative of the imino group which is easily formed is the ureide.



This is made by the addition of a salt of thyroxin, either the sodium or zinc salt, to acetic acid to which potassium cyanate has already been added. Cyanic acid reacts with thyroxin with the formation of the ureide. It separates from boiling water in curved needle form and has very closely the same solubilities as the acetyl (Fig. 4). Analysis of the ureide shows the percentage of iodine to agree with the theoretical, 60.67. This is a third confirmation of the molecular weight 585. Although thyroxin forms a stable salt with sulfuric acid which is not hydrolyzed by boiling in dilute sulfuric acid, the addition of the acetyl or ureide groups to the imino increases the acidic properties of the imino, and these derivatives do not form stable salts with sulfuric acid except at low temperatures. Boiling the sulfate in dilute sulfuric acid causes a complete hydrolysis and separation of the acetyl or ureide in free form. The presence of the imino group in thyroxin is established by identification of the indole nucleus, the formation of the acetyl and ureide derivatives, and by the power to form salts with mineral acids.

The evidence for the carboxyl and hydroxy groups is as follows: Thyroxin is extremely insoluble in aqueous solutions of all acids, including carbonic. It is very easily soluble in sodium potassium and ammonium hydroxide, but the weakness of the acidic groups

on the molecule is shown by the fact that boiling water alone causes a complete hydrolysis of the ammonium salt and free thyroxin may be precipitated in crystalline form by boiling an aqueous or alcoholic solution of its ammonium salt. Dilute solutions of sodium and potassium carbonate will dissolve only a small amount of thyroxin in the cold, but it is soluble in very dilute solutions of

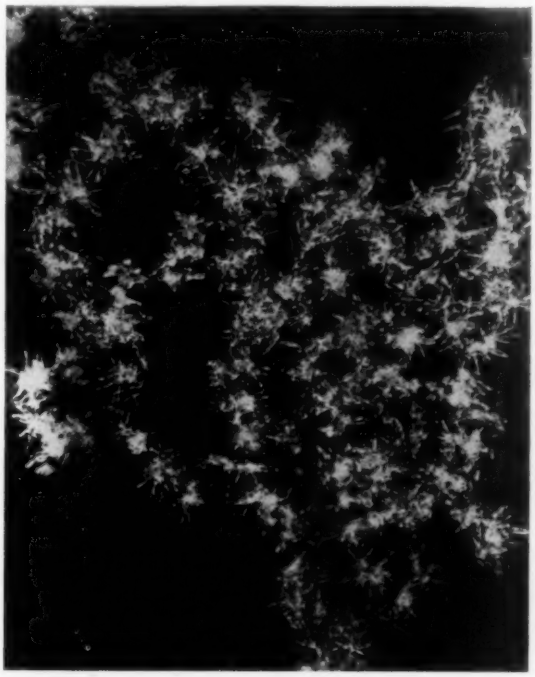


FIG. 4. The crystal form of the ureide derivative of thyroxin.

sodium and potassium carbonate at 100°C. However, on cooling such a solution, a mono-metal salt of thyroxin separates in crystalline form. If an excess of carbonate is present at first, the mono-salt of thyroxin is so insoluble in the presence of the excess sodium or potassium carbonate that most of the thyroxin being tested remains insoluble. The addition of a very slight amount of sodium or potassium hydroxide to a solution containing a

suspension of thyroxin in the presence of sodium or potassium carbonate immediately carries the thyroxin into solution. These reactions suggest that there are present in thyroxin both carboxyl and hydroxy groups. The carboxyl group reacts with carbonates but the resulting mono-salt is so slightly soluble that the presence of excess carbonate forces the mono-salt out of solution. The hydroxy group in the presence of carbonates alone does not react, but the addition of hydroxide to such a solution forms a metal salt with the hydroxy group, and the di-metal salt is readily soluble.

Still further evidence for this action is found in the barium salt. Barium chloride added to a sodium hydroxide solution of thyroxin precipitates thyroxin in needle crystals, usually twined, or in sheaves or bundles. If this is filtered off it is found to be slightly soluble in boiling water. On cooling and with the addition of a soluble barium salt to the solution, the barium salt of thyroxin recrystallizes quantitatively. If red litmus paper is dipped into a boiling aqueous suspension of the barium salt, the solution reacts neutral, but wherever the crystals of the barium salt come in contact with the paper the color of the indicator is changed to blue, showing that hydrolysis of the barium salt has occurred. If sodium hydroxide is added to an aqueous suspension of the barium salt of thyroxin the barium salt is dissolved and becomes almost as soluble as the sodium salt. This behavior is explained by the fact that the second hydroxyl group of barium hydroxide is not sufficiently strong to form a soluble salt with the hydroxy group of thyroxin. In the presence of boiling water the hydroxy group of thyroxin and one hydroxy group of barium exist in free form, barium forming a salt only with the carboxyl group in thyroxin. Both hydroxy and carboxyl groups are slowly hydrolyzed by prolonged boiling of the barium salt in water.

The difference between the two acid groups is also shown in a sodium potassium, or ammonium hydroxide solution of thyroxin. If carbon dioxide is bubbled through such a solution so as to produce sodium carbonate, but not bicarbonate, the hydroxy group is freed from metal and the mono-metal salt of thyroxin separates in flat crystals, oval, rectangular, or square. If an excess of carbon dioxide is passed through the solution, the carboxyl group also is freed and thyroxin will separate. The sepa-

ration of the monosodium salt occurs at the point where the hydroxy group has been freed, but the carboxyl group is still in the form of a salt (Figs. 5 and 6).

While endeavoring to separate the metal salts for analysis the monosodium, potassium, and ammonium salts of thyroxin were prepared by dissolving thyroxin in strong solutions of the hydroxides and passing carbon dioxide through these until the

FIG. 5. The monopotassium salt of thyroxin which separates in small flat plates, rectangular, or square.

mono-metal salt separated. The crystals were filtered on a small Buchner funnel, and washed with water. It was found that approximately 60 per cent of the amount of thyroxin taken was left on the funnel after drying in the supposed form of the monosodium, potassium, and ammonium salts. It was also found that if the salt was washed on the funnel with a 20 per cent solution of

sodium or potassium chloride, and not with water, the compound did not melt. When, however, the mono-salt was washed with water the sodium, potassium, and ammonium salts all had the same melting point, 204°. Since the mono-salt of thyroxin does not melt and the sodium, potassium, and ammonium salts, washed with water, all melt at exactly the same point, it seemed probable

FIG. 6. The monoammonium salt of thyroxin which separates in long blades.

that the washing with water was sufficient to hydrolyze the very weak carboxyl, with the result that free thyroxin was left on the paper, the base being entirely washed away. In order to determine this the monoammonium salt was prepared as above, filtered, and washed with water and then analyzed for ammonia by means of Nesslerization. Nesslerization, although an exceedingly sensitive test for ammonia, failed to show the presence of even the

faintest trace of ammonia in the supposed monoammonium salt of thyroxin. It was therefore evident that by washing the monosodium, ammonium, and potassium salts of thyroxin with water the weak carboxyl group can be completely hydrolyzed, and since the hydroxy group already was in the free form, the molecule existed with both carboxyl and hydroxy groups uncombined with metal.

FIG. 7. The disilver salt of thyroxin which separates in large, flat, rectangular and square plates, often occurring in sheaf form and twined.

Evidence that a di-metal derivative of thyroxin does form is furnished by the silver salt. Although a disilver salt containing the theoretical amount of iodine has not been prepared, this salt has been made with so much silver present that it amounted to 92 per cent of the theoretical for the addition of two atoms of silver to the molecule. The reason why the theoretical disilver salt

cannot be prepared is undoubtedly due to the weakness of the hydroxy group (Fig. 7). When the disilver salt, which is highly crystalline, is washed in order to remove the excess of silver nitrate and ammonia which are used in its formation, the hydroxy group hydrolyzes to some extent, and the amount of silver remaining is slightly less than theoretical.

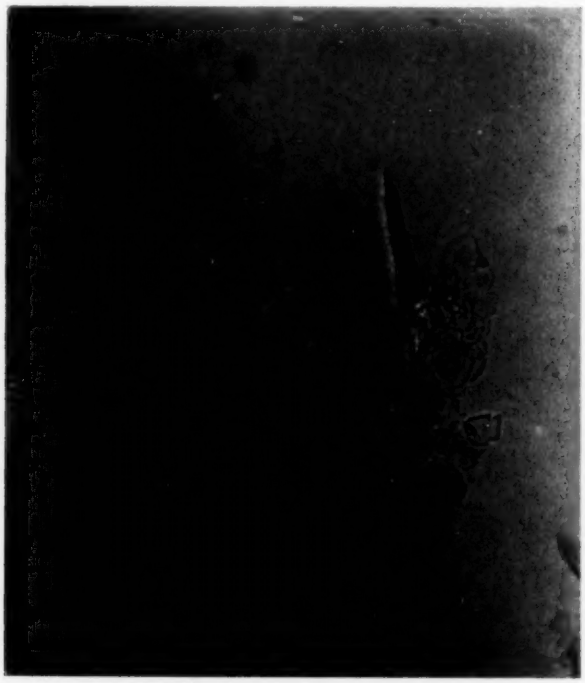


FIG. 8. The dipotassium salt of thyroxin which separates in flat plates with rough, irregular edges.

Di-basic salts of sodium, ammonium, and potassium are formed by dissolving thyroxin in the respective hydroxides, and adding a corresponding salt of the alkali preferably the chloride until the di-alkali salt of thyroxin becomes insoluble and precipitates in crystal form (Fig. 8). Di-basic salts, which are only slightly soluble, have also been prepared with barium, calcium, magnesium, nickel, zinc,

and copper (Fig. 9). Although all these salts may be made in beautifully crystalline and characteristic form, it is impossible to filter and separate them in a high state of purity by washing with water. Just as hydrolysis of the hydroxy group caused a lower silver content than theoretical with the silver salt, the hydrolysis of the hydroxy group with the barium salt shows a lower percentage

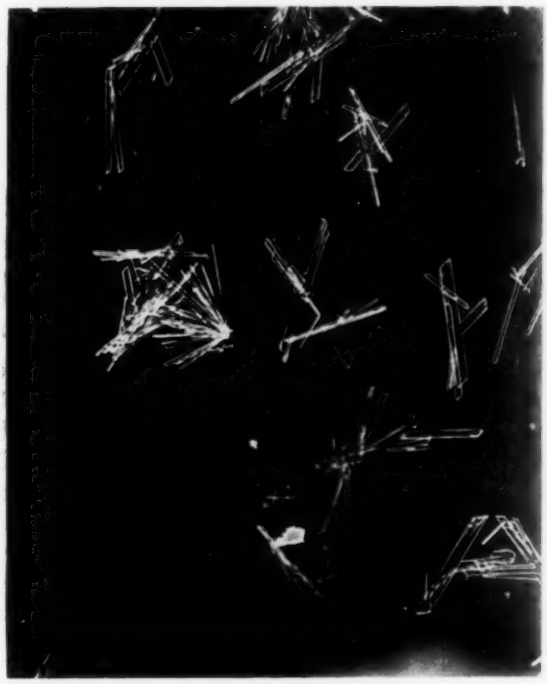


FIG. 9. The zinc salt of thyroxin which separates as long, flat blades in bundles and rosettes.

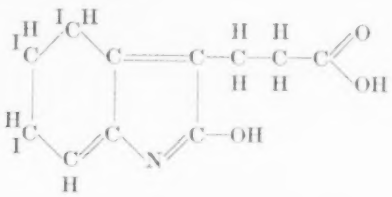
of iodine than theoretical. However, the weight of barium added is about twice that required for the mono-metal salt. With the less basic properties of calcium, magnesium, zinc, nickel, and copper, even greater differences occur between the amount calculated for a di-metal salt and the amount found. But in every case since the amount of metal present nearly agreed with that required

for a di-basic salt, the possibility that these are mono-basic salts is excluded. All salts of thyroxin which are insoluble in water such as the silver, copper, zinc, nickel, calcium, and magnesium are soluble in sodium hydroxide. The solubility is probably due to the same reactions that occur with the barium salt. The weak basic properties of the metals are insufficient to form soluble di-basic salts, but sodium hydroxide carries the salt into solution by adding to the hydroxy group of thyroxin.

Other evidence for the carboxyl and hydroxy groups is furnished by the dimethyl ester. Methyl iodide added to an alcoholic suspension of the silver salt forms the dimethyl ester. This is soluble in alcohol but insoluble in water even in the presence of sodium hydroxide. By heating in dilute alcoholic sodium hydroxide, hydrolysis of the methyl ester of the carboxyl occurs and the oxymethyl derivative is obtained.

Tautomeric Forms of Thyroxin.

Thyroxin reacts in the presence of alkalies forming dibasic salts, but differences between the two acidic groups indicate that one is a carboxyl and one a hydroxy group. When thyroxin exists in this form, which will be called the enol, the hydroxy group is adjacent to the nitrogen but there is a double-bond between the nitrogen and the alpha carbon, and no hydrogen is attached to the nitrogen.



In acid solution thyroxin forms derivatives which demonstrate the presence of an imino group and exists in its tautomeric form, with imino carbonyl groups adjacent. This will be called the keto form. When thyroxin was first isolated it was in the keto form and although it seemed probable that the hydrogen migrated in alkaline solution with a change from carbonyl to hydroxy groups, no quantitative data were available for proof of the hypothesis.

When the mono-metal salts of sodium, ammonium, and potassium were prepared by freeing the hydroxy group in alkaline solution with carbon dioxide, it was found that by washing with water complete hydrolysis of the carboxyl also occurred. When the hydrolysis of the ammonium salt was carried out at 100° both acidic groups were not only freed but, in addition, the boiling water caused

FIG. 10. The crystals of the enol form of thyroxin.

a change from the enol to the keto form. However, if the mono-metal salts are hydrolyzed with cold water the enol form is retained. There are many differences in the chemical properties of the enol and keto forms, but the most striking difference is in the melting point. The melting point of the enol form is 204°, that of the keto, 250°.

When the enol form of thyroxin was prepared by cold hydrolysis of its ammonium salt, it still retained the crystal form of this salt,

but by dissolving the crystals in pyridine and adding water the enol form separated in its own characteristic crystal form (Fig. 10). The enol form of thyroxin separates in needle crystals which are much shorter than those of the keto form and always occur in rosettes or sheaf-like bundles. Crystallization does not alter the melting point.

FIG. 11. The simultaneous crystallization of both enol and keto forms of thyroxin from an aqueous pyridine solution.

The keto form of thyroxin is by far the more stable and, unless precautions are observed, the enol form readily passes over into the keto. The most important factors influencing the change from enol to keto form are the presence of water and the hydrogen ion concentration. By adding water to a pyridine solution of the enol form, conditions may be produced in which both enol and keto forms simultaneously crystallize (Fig. 11). On long standing even

at room temperature, the enol form slowly changes over to the keto and the keto form alone separates. Since the chief chemical properties of thyroxin are due to its basic and acidic groups, a brief summary of solubilities and reactions of the two tautomeric forms is of interest.

The enol form of thyroxin is much more soluble than the keto, and the solubility may be used as a test of the form in which thyroxin is present. For example, the keto form is insoluble in all organic solvents, such as all alcohols, ether, chloroform, ethyl acetate, acetone, carbon disulfide, quinoline, pyridine, anhydrous or aqueous, and aniline. The enol form is readily soluble in anhydrous or aqueous pyridine. Therefore, pyridine alone is not sufficiently basic to change the keto into the enol form, but when this change has been produced pyridine readily dissolves thyroxin. Since ammonium hydroxide in water, alcohol, or pyridine will change the keto form to the enol, but pyridine cannot produce this change, the hydroxyl ion concentration necessary for the conversion from one tautomeric form to the other lies between the basicity of dilute ammonium hydroxide and that of pyridine. Since the enol changes to the keto in a boiling aqueous pyridine solution, the acidity necessary for the tautomeric change in this direction lies between the narrow limits of the hydrogen ion concentration of a cold and a boiling aqueous solution of pyridine. The limits for the change in tautomeric form are at the same time the limits of solubility for thyroxin in alkaline solution. Thyroxin is soluble in enol form, in pyridine and quinoline, but any higher concentration of hydrogen ion causes the change to the keto form and limits the solubility of thyroxin in alkalies. Thyroxin in the keto form remains insoluble in all organic solvents with hydrogen ion concentrations equal to or less than that of glacial acetic acid. It is soluble in formic acid, but the subsequent addition of water causes thyroxin to separate again. Although acetic acid will not make thyroxin soluble in alcohol the addition of a mineral acid renders thyroxin readily soluble in alcohol. Solubility under these conditions is evidence for the formation of salts with the imino group.

The acid and basic properties of thyroxin therefore lie between these two limits: (1) The formation of salts with acids through the imino group of the keto form with formic acid, but not acetic;

and (2) the formation of salts with alkalies through both the carboxyl and hydroxy groups with dilute ammonia, but not with pyridine. Lying between these two limits are the formation of mono-metal salts through the carboxyl alone in the presence of carbonates but not bicarbonates, and finally the complete precipitation of thyroxin from the alkali metal salts by carbonic acid or by hydrolysis with water in a boiling ammoniacal solution. The mono-metal salts of thyroxin are but slightly soluble in water but are easily soluble in alcohol. The solubility in alcohol is due to the fact that although only the carboxyl group is combined with metal, the molecule is in the enol and not the keto form.

The imino group of thyroxin reacts with all acids stronger than and including formic acid, but no acid salt of thyroxin is appreciably soluble in water, and even the sulfate which is the most soluble is only very slightly so. The great insolubility of the keto form of thyroxin is one of the most important factors permitting the isolation of the compound. The insolubility of thyroxin is also of importance in a consideration of its chemical properties. As soon as the proper conditions exist in any solution for the formation of the keto form of thyroxin, the reactive groups are thrown almost completely out of the sphere of reaction by the insolubility of the compound.

The keto form of thyroxin is soluble in organic solvents only when some acid is present which is capable of forming an acid salt with the imino group. Thyroxin may be conveniently purified by dissolving either in alkaline alcohol with the addition of acetic acid, or by dissolving in acid alcohol with the addition of sodium acetate. In the presence of acetic acid the imino group does not form a salt and thyroxin precipitates in needle form.

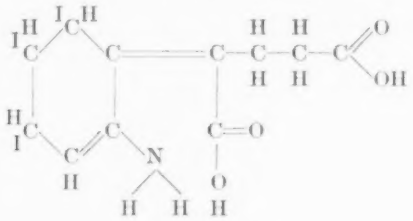
Open-Ring Form of Thyroxin.

Since thyroxin in keto form is insoluble in bases weaker than ammonium hydroxide and is insoluble in alcohol in the presence of acids weaker than formic, there is a wide range of hydrogen ion concentration in which pure thyroxin is insoluble. These limits of solubility, however, apply only to pure thyroxin in aqueous and alcoholic solutions. In the presence of certain substances, changes in the acid and basic groups occur and the solubilities of

thyroxin are materially altered and extended. In the presence of the products resulting from alkaline hydrolysis of the thyroid proteins the solubility of thyroxin is so greatly altered that it is completely soluble in carbonic acid, and even acetic acid produces an incomplete precipitation. Hydrochloric and sulfuric acids precipitate thyroxin under these conditions, but in excess they redissolve a considerable percentage of the total amount present. The increased solubility in acids indicates an increase in the strength of the basic groups of the thyroxin molecule. Although pure thyroxin is practically insoluble in pyridine, sodium carbonate, barium hydroxide, and alcohol, partially purified thyroxin is readily soluble in the presence of all these reagents. The increased solubility in weak alkalies indicates an increase in the strength of the acidic groups in the thyroxin molecule. These changes in the chemical properties of thyroxin are most marked during the early stages of purification while there is a large percentage of impurities present, but that the alteration is due entirely to the impurities is disproved by the fact that both increased acidic and basic properties persist even after the removal of all but a trace of the impurities. Furthermore, the addition of certain substances to a solution of pure thyroxin brings about a similar increase in both basic and acidic properties. The solubility of partially purified thyroxin in weak alkalies is in such striking contrast to the solubility of pure thyroxin that it cannot be explained except by a change in the structure of the molecule other than the two tautomeric forms described above. The exact nature of this change was suggested by a study of the acetyl.

In all derivatives of thyroxin involving the hydrogen of the imino group, it is impossible to make the enol form, as the hydroxy group cannot exist. Because of the absence of the hydroxy group these derivatives should form mono-basic salts through the carboxyl group alone, they should be more insoluble in alkalies, and should form insoluble barium and silver salts. Since acid salts of the imino are soluble in alcohol, acetic acid, and ethyl acetate, it seemed probable that derivatives attached to the imino would also make the molecule soluble in these reagents. After the acetyl and ureide were prepared in pure form they were found to be easily soluble in alcohol, acetic acid, and ethyl acetate, but instead of being less soluble in alkalies, the acetyl was more soluble and could be held in solution with as weak a base as pyridine alone.

The acetyl was not only more soluble in weak organic bases but it also formed a silver salt which was completely soluble in dilute ammonium hydroxide. The silver salt of thyroxin will separate from strong ammonium hydroxide, but the silver salt of the acetyl is so soluble that it is impossible to prepare it in the presence of ammonia. By dissolving the acetyl in pyridine, however, the addition of silver nitrate produces a voluminous precipitate which may be washed, dried, and analyzed. When this was done it was found that the acetyl had formed a di-basic salt with silver. The formation of a di-basic salt by the acetyl indicated the presence within the molecule of another acidic group other than the terminal carboxyl. The simplest change by which a carboxyl group could be formed would be by introduction of a molecule of water between the imino and carbonyl groups, changing the imino carbonyl groups to amino carboxyl groups, and in the case of the acetyl the amino group is combined with one acetyl radical.



The presence of the acetyl radical in place of the imino hydrogen prevents the tautomeric change to the enol form, but in place of this, the ring opens even in the presence of weak organic bases. The acetyl attached to the amino group, however, does not prevent the closure of the ring, and if an alkaline solution of the acetyl, which is present in the open-ring form, is added to a dilute mineral acid at 100°, the ring closes and the acetyl separates in crystalline form. In addition to the disilver salt of the acetyl, the zinc salt has been made, and di-basic sodium, ammonium, and potassium salts of the open-ring form of the acetyl may be prepared by dissolving in the respective hydroxides and adding a corresponding salt, the chloride or acetate, until the salt of the acetyl becomes insoluble (Figs. 12 to 15). If sodium hydroxide and sodium acetate are used, very large, flat, jagged plates result;

with sodium hydroxide and sodium chloride short needle crystals are formed.

Barium and calcium salts of the acetyl can also be formed by the addition of barium or calcium chloride to a solution of the acetyl in dilute sodium hydroxide or pyridine. An excess of pyridine dissolves the salt.

FIG. 12. The disodium salt of the acetyl derivative separated from a sodium chloride solution.

After the physical and chemical properties of the acetyl derivative had been established, it was found that a most striking resemblance existed between partially purified thyroxin and the acetyl. The acetyl differs from thyroxin in having wider limits of solubility in weak bases, and the greatest difference between partially purified and pure thyroxin is the solubility of the former in weaker alkalies as sodium carbonate, barium hydroxide, pyridine,

and in alcohol. These reactions suggested that in partially purified thyroxin the structure of the molecule is similar to that of the acetyl. But the possibility that an acid radical was attached to the imino group, as in the acetyl, could be excluded by the fact that the thyroxin could be separated in keto form. The increase in both acidic and basic properties, its close resemblance in chemical

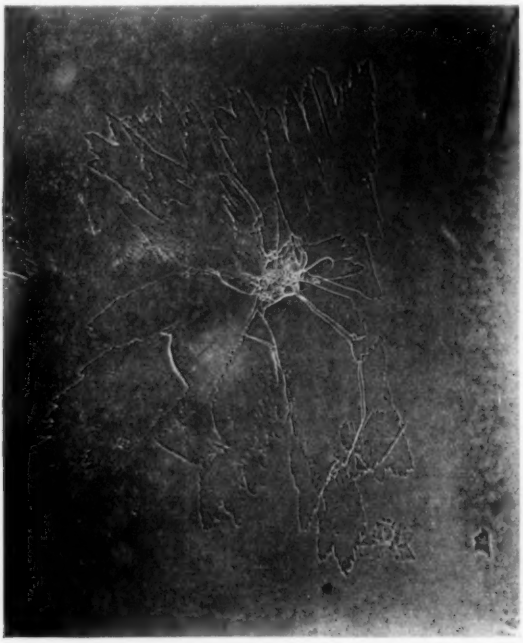


FIG. 13. The disodium salt of the acetyl derivative separated from a sodium acetate solution.

reactions to the acetyl, and the fact that it could be separated in the keto form suggest that in partially purified thyroxin the molecule is present neither in the keto nor enol forms, but that the pyrrole ring exists in open form, the elements of water entering between the carbonyl and imino groups.

This structure of the molecule of thyroxin will be called the open-ring form. The open-ring form of indole derivatives con-

taining an alpha carbonyl group is of common occurrence, but thyroxin is perhaps unique in the great ease with which the ring opens and the great difficulty with which the ring closes in the presence of certain substances.

Although the open-ring structure of thyroxin was first suggested by a study of the acetyl, further investigation has amply confirmed this hypothesis, and brought to light the delicately balanced

FIG. 14. The dipotassium salt of the acetyl derivative.

reactions, which, in all probability, are involved when the substance functions physiologically. These reactions are concerned with the opening and closing of the ring and the formation of salts with acids by the amino and imino groups. When sulfuric acid is added to a slightly alkaline alcoholic solution of thyroxin and the alcohol is distilled, the sulfate of thyroxin separates, the sulfate radical being attached to the imino group. However,

if sulfuric acid is added to an alkaline aqueous solution of thyroxin, the resulting precipitate is not the imino sulfate of thyroxin. Analysis of this precipitate for its iodine content showed that thyroxin had not precipitated in free form but contained one equivalent of acid. Further investigation showed that thyroxin precipitates with one equivalent of acid, not only with sulfuric

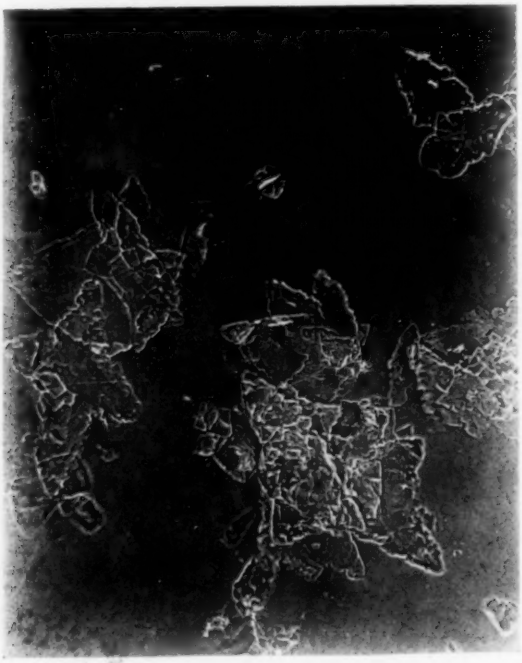


FIG. 15. The dipotassium salt of the ureide of thyroxin which separates in a manner similar to the dipotassium salt of the acetyl derivative.

but with weak organic acids and that even carbonic acid adds to thyroxin when carbon dioxide is passed through an alkaline solution. The sulfate, chloride, phosphate, trichloracetate, oxalate, formate, acetate, and carbonate of thyroxin have been prepared. All these salts are soluble in alcohol and have melting points which are strangely similar, all of them melting at about 204°. Although the imino group of thyroxin in keto form is so feebly

basic that in hot dilute hydrochloric solution the acid radical is hydrolyzed and the imino group exists in free form, when the enol form of thyroxin is precipitated by an acid, an equivalent of acid is contained in the precipitate attached to thyroxin. If any of these salts, prepared by acidifying an alkaline solution of thyroxin, are removed from solution, suspended in distilled water, and boiled, a change occurs, and thyroxin

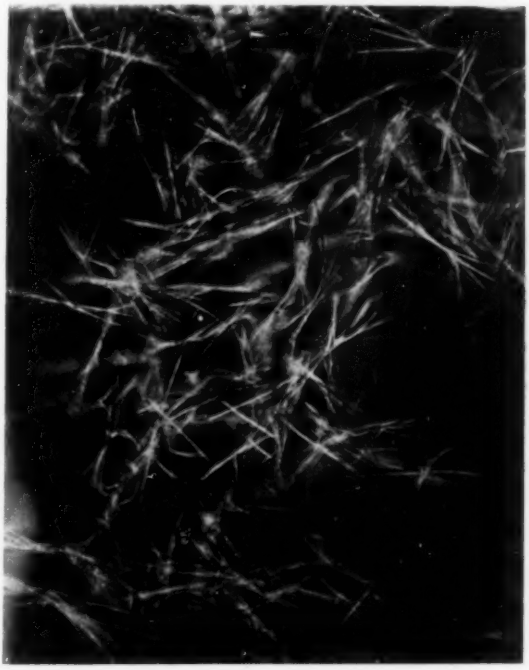
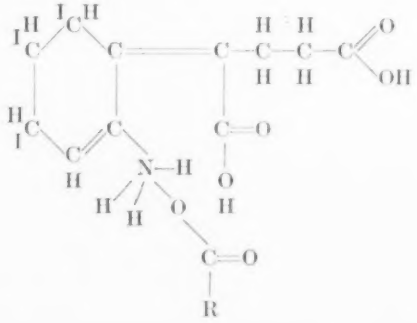


FIG. 16. Crystals of thyroxin in the amino carboxyl form.

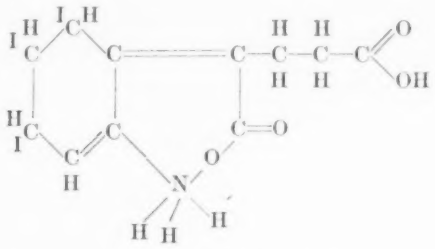
precipitates in long, bundle blades. These blades differ from the keto form of thyroxin in being soluble in alcohol and having a melting point of 225°. If instead of suspending the acid salts in distilled water, they are added to a dilute solution of hydrochloric or sulfuric acid and are then boiled, the keto form of thyroxin separates. These reactions are interpreted as follows:

When an acid is added to the enol form of thyroxin, the nitrogen becomes pentad and the acid radical adds to the nitrogen. In

aqueous solution the pyrrole ring is no longer stable and the elements of water add between the pentad nitrogen and the carbonyl group forming a carboxyl group and an acid salt of the amino group.



In cold water solution this reaction occurs not only with sulfuric and strong organic acids, such as trichloracetic and oxalic but even carbonic acid is capable of adding to the amino group. When the amino salt is suspended in distilled water and boiled, the amino group is hydrolyzed free from the acid, and the carboxyl group which is adjacent to the amino group forms a salt with the amino group. The compound then exists in an amino carboxyl salt form, the acid used in precipitating thyroxin having been expelled from the amino group by hydrolysis with water.



This form of thyroxin differs from both the keto and enol forms in having the addition of the elements of water. It has a melting point of 225° and is soluble in alcohol (Fig. 16). It is converted into the keto form very easily, merely solution in alcohol

is sufficient to expel the water, and the keto form of thyroxin then separates. It is impossible to separate the amino carboxyl salt form of thyroxin from solutions containing a high percentage of alcohol. Further investigation showed that this is also true of pyridine and other organic solvents. It is necessary to have water present in order to force the opening of the pyrrole ring.

If any acid salt of the amino group of the open-ring form of thyroxin is suspended in distilled water and boiled, the carboxyl

FIG. 17. Crystals of the amino carbonate of thyroxin.

group in thyroxin, which is adjacent to the amino, will displace the acid radical attached to the amino, and the amino carboxyl salt form of thyroxin results. If the acid radical which is added to the amino is sufficiently strong and an excess of the acid is present, the ring does not remain open, but the elements of water are expelled and the strong acid radical is either hydrolyzed from the imino group or remains attached as an acid salt of the imino group. The closing of the ring of an amino-acid salt is influenced by many factors, such as the amount of acid present, the strength of the acid, and the presence of organic solvents, such as pyridine

or alcohol. Strong acids promptly close the ring, forming imino salts; weak acids are expelled from the amino, and the molecule exists in the amino carboxyl form. The presence of organic solvents such as alcohol results in the closing of the ring and the formation of imino salts with strong acids, or the displacement of the acid with the separation of thyroxin in keto form. If an

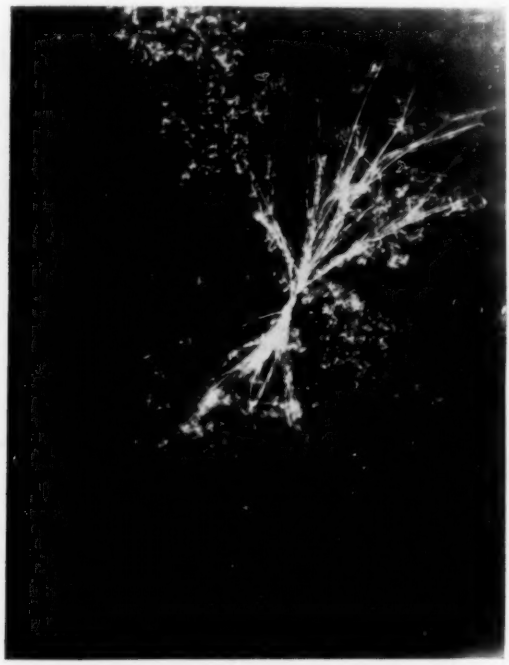


FIG. 18. A mixture of the crystals of the amino formate changing into the amino carboxyl salt form of thyroxin. The long needles are the amino carboxyl crystals.

amino-acid salt of the open-ring form is washed free from all acid, suspended in neutral water, and boiled, the acid is promptly hydrolyzed from the amino group and the amino carboxyl salt form results, but in the absence of excess acid in solution this form of thyroxin is unstable at 100°C., water is rapidly expelled from the ring, and the keto form of thyroxin separates in very

fine, thread-like crystals. Under proper conditions all three forms may be present at the same time (Figs. 17 to 19).

Since acids added to the enol form of thyroxin in aqueous solution cause an opening of the ring, the question arose as to whether the opening of the pyrrole ring is the primary action, or whether it is secondary to the existence of the nitrogen in the pentad form. That the opening of the ring occurs without passing

FIG. 19. A mixture of amino carboxyl crystals changing into the keto form of thyroxin. The long needles are crystals of the amino carboxyl form of thyroxin.

through the enol form is shown by the formation of the amino sulfate directly from the imino sulfate (Figs. 20 and 21). When the imino sulfate is present in a small amount of alcohol and water is added, the ring opens and the amino sulfate separates even though the molecule had existed in the keto form. The formation of amino salts from imino salts shows that the ring opens

readily when the nitrogen is in the pentad state, but that the opening of the ring also occurs directly from the enol form in a solution slightly alkaline may also be shown. When the disodium salt of thyroxin is dissolved in cold water and ammonium chloride is added to the solution, the sodium is hydrolyzed from hydroxy and carboxyl groups, resulting in the precipitation of thyroxin in the enol form. If this suspension of the enol form is now boiled

FIG. 20. The imino sulfate of thyroxin separated from a hot solution.

the crystal form changes into the typical amino carboxyl salt form, the melting point of which is 225°. The crystals are also readily soluble in alcohol which excludes the possibility of their being in the keto form. A more direct evidence of the existence of thyroxin in open-ring form is obtained by dissolving the disodium salt in hot water and adding ammonium chloride to this hot solution. Instead of thyroxin separating in the enol form, it

separates directly as the amino carboxyl salt form. The most important factor in this reaction is the presence of excess alkali. If much alkali is present, the molecule exists in the enol form and addition of ammonium chloride will cause a separation of the monoammonium salt.

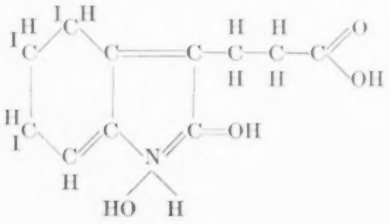
From these results it would appear that in acid alcohol solutions thyroxin exists in the keto form. In the presence of excess alkali

Fig. 21. The amino sulfate of thyroxin formed from the imino sulfate of thyroxin by the addition of water to an alcoholic solution of imino sulfate.

in aqueous solution thyroxin exists in the enol form, but as the neutral point is reached from either direction there is a tendency for the ring to open. In a hot neutral solution the ring does open. In a cold neutral solution even carbonic acid will open the ring and add to the amino group.

The Amino Hydrate Form of Thyroxin.

The pyrrole ring of thyroxin not only has a tendency to take up water between the imino and carbonyl groups and exist in amino carboxyl form, but the amino group is so strongly basic that in a slightly alkaline solution the elements of water will add to the nitrogen forming the amino hydrate.



This form of thyroxin is tautomeric with the amino carboxyl form. It is very readily prepared by heating an alkaline solution of thyroxin, removing the solution from the flame, and adding 10 per cent ammonium chloride. The solution becomes turbid, and fine branching crystals separate (Fig. 22). The melting point of this form of thyroxin is 216°. If these crystals are suspended in distilled water containing a small amount of formic acid and the solution is boiled, the crystals are changed into their tautomeric amino carboxyl form, whose melting point is 225° (Figs. 23 and 24).

One form of thyroxin changes into another so easily that enol and keto forms will crystallize simultaneously out of the same solution, and amino-acid salt, amino carboxyl salt, and keto forms may all be present at the same time, one form changing into another as the boiling of the solution is continued. The ready change of thyroxin from one form to another is explained by the great ease with which the pyrrole ring opens and the elements of water are added to the molecule. This reaction does not occur with indole or isatin and was not at first easily explained in the case of thyroxin. While engaged in the preparation of the intermediate products for the synthesis of thyroxin, our attention was drawn to the fact that the explanation of the peculiar properties of the imino group in the pyrrole ring of thyroxin is the presence

of the hydro groups in the benzene ring. Analine is a feeble base and the imino group in indole is still more feebly basic. Hexahydro-analine has such a strongly basic amino group that it will combine with carbon dioxide from the air, and it has a very caustic action on the skin. It is the addition, therefore, of four hydro groups to the molecule that so modifies the nucleus giving basic

FIG. 22. The crystals of the amino hydrate form of thyroxin.

properties to the imino group of the pyrrole ring of thyroxin. Speculation as to the properties of the compound in which the three iodines are replaced by three hydrogens may be deferred until the substance is prepared synthetically, but that the imino group of the pyrrole ring will be still more basic in this compound would naturally follow from the general law that addition of halogen to the benzene ring renders the ring more acidic. The

position and reason for the three extra hydrogens in thyroxin were unknown and were very puzzling until the reactions of the compound involving the amino and imino groups caused the necessity of explaining this action by some modification of the indole nucleus. Since the introduction of the six hydro groups in analine greatly increases the basicity of the amino group, the addition of four hydro groups to the indole nucleus of thyroxin is an adequate

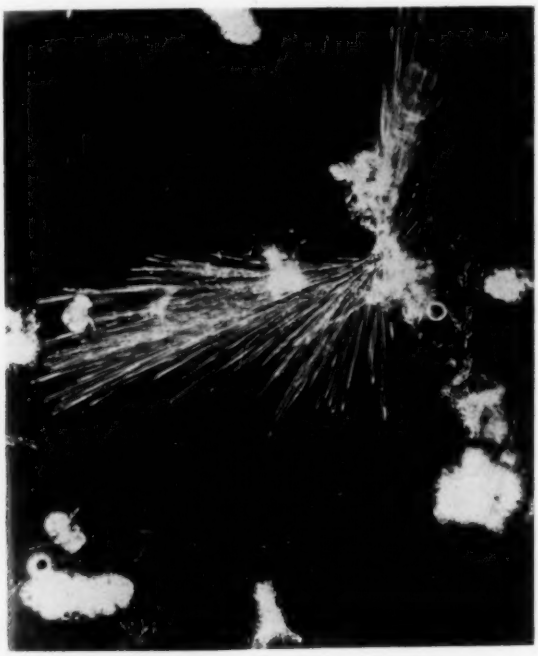


FIG. 23. A mixture of enol and keto forms of thyroxin crystallizing simultaneously from the same solution.

explanation of the increased basicity of its amino group. The instability of the pyrrole ring of thyroxin in contrast to that of indole, and other unreduced derivatives of pyrrole is due to the increased basic properties of the nitrogen in thyroxin. This point is well illustrated in the stability of the amino carboxyl form. In neutral solution the nitrogen tends to become triad, the pyrrole

ring is more stable than the amino carboxyl salt, and thyroxin separates in keto form. If a slight amount of acid is present, the nitrogen remains in the pentad state and the amino carboxyl form is so stable that it is impossible to expel water from the molecule and make the keto form. The difference in the basicity of the nitrogen when changing from the open- to the closed-ring forms is probably involved when thyroxin functions physio-

FIG. 24. A mixture of the crystals of the amino carboxyl form changing into the keto form. The small rosettes are crystals of thyroxin in the keto form.

logically. But the unique chemical properties of thyroxin are also due in large measure to the carbonyl group adjacent to the imino, and the reactivity of the substance *in vivo* and *in vitro* is due to the presence of this oxy group in the indole nucleus. It was for this reason that the compound was named thyr-oxy-indole or thyroxin.

After it was found that thyroxin forms amino salts with feeble carboxyl groups, it was of especial interest to form the amino salt of thyroxin with glycine. Reserving a study of the reaction between thyroxin and the amino-acids for a further communication, merely the formation of an amino-acid salt between thyroxin and glycine will be reported in this paper.

Since acid added to the enol form of thyroxin results in the formation of an amino salt, it seemed probable that at least a portion of the nitrogen of thyroxin should react as amino nitrogen with nitrous acid when the molecule existed in the amino-acid salt form. This was tried and it was found that when an alkaline aqueous solution of thyroxin was added to a Van Slyke amino-acid apparatus, approximately 70 per cent of the total nitrogen present was liberated as amino nitrogen. When the keto form of thyroxin was used, no nitrogen was evolved. When the amino carboxyl form of thyroxin is added to nitrous acid, about 15 per cent of its total nitrogen is evolved as amino nitrogen. The reason that a quantitative evolution of amino nitrogen does not occur with the last mentioned form in 3 minutes is because the crystals are insoluble and the reaction takes place at a very slow rate.

When nitrous acid is added to an alcoholic solution or to an aqueous suspension of thyroxin in the presence of hydrochloric acid, a yellow color is produced. Upon the addition of ammonia this is changed to a deep red which in dilute solution is pink. This color reaction is convenient for a rough qualitative test for thyroxin. However, if acetic or sulfuric acid is used in place of hydrochloric, a fainter yellow color is produced, and the addition of ammonia gives a yellowish orange instead of a red color.

During the purification of thyroxin, the presence of colloidal impurities is sufficient to cause the opening of the ring and also to prevent the closing of the ring. When an alkaline solution of thyroxin is acidified the precipitate carries down many of the impurities present as salts of the amino group and hence no quantitative separation can be effected by precipitation with an acid. The chief problem in the isolation of thyroxin is to close the ring in the presence of the impurities, and thereby produce chemical properties specific to the thyroxin molecule, which permit of a separation. This difficulty in closing the open-ring form of thyroxin is well illustrated in the course of its purification. Approxi-

mately 50 per cent of the iodine content in the early steps of the separation of thyroxin is soluble in barium hydroxide. This barium-soluble portion may be hydrolyzed by heating with barium hydroxide for many hours, precipitated with acid, given another treatment with barium hydroxide, and this process continued as many as seven or eight times without rendering thyroxin insoluble in barium hydroxide. This treatment, however, slowly separates many of the impurities and the percentage of iodine in the dry material may reach as high as 58 per cent. Thyroxin in this open-ring form contains sufficient impurities to impart a distinctly yellow color, and it is readily soluble in sodium carbonate, barium hydroxide, pyridine, and alcohol. By chance such a preparation was dissolved in sodium carbonate solution and was allowed to stand 7 weeks. At the end of that time a white residue had separated and settled to the bottom of the flask. Examination showed this to be the monosodium salt of thyroxin. Although the material was in the open-ring form when dissolved in the carbonate, on long standing the ring had closed and the compound thereby became insoluble in sodium carbonate.

The open-ring form of thyroxin cannot be precipitated from alcohol with acetic acid. The keto form of thyroxin is very nearly quantitatively precipitated from alcohol by acetic acid, but as long as impurities are present, an alcoholic solution of the open-ring form of thyroxin may be allowed to stand several weeks without any trace of thyroxin separating. If, however, an alcoholic solution of thyroxin is slowly evaporated on the water bath, the evaporation causes a partial separation. A yellow oily tar creeps up the inclined bottom of the evaporating dish and forms a ring as the alcohol evaporates. At the spot where the last trace of alcohol was left, a dry crusty material, which is almost white, shows the partial separation of thyroxin in the keto form. This property of thyroxin to separate from alcohol, even in the presence of impurities, is the reaction by which thyroxin was first isolated. The alcohol in this case was evaporated unintentionally, and, although the entire sample of thyroxin had been completely soluble in the alcohol, the slow evaporation and subsequent heating at 100° was sufficient to close the ring in a small percentage of the total amount with the result that it was insoluble on the addition of more alcohol. This method of sepa-

ration is not of great value for the isolation of the compound. The best method so far determined for the closing of the ring is to dissolve thyroxin in alcohol containing sodium hydroxide, and pass carbon dioxide through the solution, freeing both hydroxy and carboxyl groups. Most of the sodium carbonate is insoluble and is removed by filtration. The alcohol is distilled, leaving an aqueous sodium carbonate solution of thyroxin but still in open-ring form. Allowing this to stand for several days will cause a separation of the monosodium salt in the enol form which may be purified by similar treatment.

Oxidation and Reduction of Thyroxin.

Quantitative oxidation and reduction experiments with thyroxin have not been carried out because of the amount of material which would be required in order to isolate the products. Thyroxin is more susceptible to reduction than to oxidation. Zinc in alkaline or acid solution breaks off iodine and appears to alter the organic nucleus. Thyroxin is reduced when heated in the presence of any metal in alkaline solution other than nickel and the heavy metals, silver, gold, and platinum. Thyroxin is stable in the presence of mild oxidizing agents. Hydrogen peroxide produces no immediate effect and in a cold acid suspension the molecule will resist oxidation with potassium dichromate or iodic acid. Potassium permanganate or bromine in hot aqueous solution causes the breaking down of the molecule. Benedict's copper solution, for the determination of sugar, causes an oxidation of thyroxin in the presence of sodium hydroxide. In the presence of ammonium hydroxide alone, thyroxin is stable in Benedict's solution heated to boiling. Free iodine if added to an alkaline solution produces a precipitate and apparently brings about a deep-seated reaction within the molecule. In acetic acid or acid alcohol, iodine has very little, if any, effect on thyroxin even at the temperature of boiling acetic acid. Further investigation showed that, in the presence of iodine, thyroxin is stable in the keto form, but not in the enol form. The changes produced by oxidation with chlorine, bromine, and iodine, have not been determined, but in alkaline solutions yellow tarry products are formed. Since the enol form of thyroxin is so

much less stable than the keto, the weakness in the molecule appears to be in the linkage of the nitrogen, and the point of cleavage is probably between the nitrogen and the hydroxy groups. When the imino carbonyl groups are present, the molecule is much more resistant to oxidation by halogen.

Another point of weakness within the molecule exists in the benzene ring. The completely reduced benzene ring readily passes over into a six carbon, straight chain form. Hexa-hydrophenol may be readily oxidized to adipic acid by the action of dilute nitric acid. In thyroxin the benzene ring is in the tetra-hydro form, and it is highly probable that the one double-bond which is present will break down with the formation of an open chain structure. A reaction similar to this is found in the oxidation of the tetra-hydro-benzene ring of sedanonic acid (2) to straight chain acids.

Due to the weakness of the linkages to the nitrogen in thyroxin, it is impossible to hydrolyze derivatives from the imino group. After the acetyl radical has been attached to the imino group, it cannot be hydrolyzed with sodium or potassium hydroxide. When treated with alkali, the compound precipitates as a di-metal salt, and is thrown out of solution. If sufficiently drastic action is applied to bring about the hydrolysis, disruption of the molecule occurs.

Thyroxin upon exposure to the sunlight in weak alkaline solution is very unstable. Within 24 hours the solution changes from colorless to a pink, or faint yellow, which deepens on standing to a brown color, depending on the amount of thyroxin present. Simultaneously with the discoloration a distinct aromatic odor is produced slightly resembling that of nicotine. Such a solution, when tested for iodine by means of starch in acid solution, shows that no iodine has been broken off in the free form. If, however, a small amount of potassium iodide is added, iodine is immediately liberated, which indicates that the iodine within the thyroxin molecule was not broken off either as hydriodic acid or as iodine, but in the form of hypoiodous acid. On longer standing a test for free iodine is given without the addition of potassium iodide and the amount of hypoiodous acid is much reduced. After several weeks no test for iodine or hypoiodous acid is given, but all the iodine is found in the form of hydriodic

acid. The reduction of the hypoiodous acid to hydriodic acid is probably brought about by the hydroindole nucleus. The finding that iodine is broken off from the thyroxin molecule in the form of hypoiodous acid and not as hydriodic has direct bearing on the physiologic action of the molecule within the body.

The Acetyl Derivative.

With the acetyl, sunlight produces a similar reaction, but in this case the solution is found to contain both hypoiodous acid and iodine. The acetyl derivative, therefore, is more susceptible to oxidation than thyroxin, and brings about a much more rapid reduction of the hypoiodous acid to iodine and hydriodic acid.

So unstable is the acetyl under certain conditions that there is a spontaneous liberation of iodine from the molecule and the simultaneous oxidation of the organic nucleus, resulting in a change of color and the production of a yellow tarry material. The conditions under which it is produced appear to be in a solution of approximately the neutrality of distilled water. If barium or calcium chloride is added to a sodium hydroxide solution of the acetyl, and the solution is boiled, no decomposition of the barium or calcium salts occurs. If magnesium chloride is used in place of barium or calcium, the basicity of magnesium hydroxide is insufficient to prevent the decomposition of the acetyl, and there is a spontaneous liberation of iodine accompanied with production of a blue color, which changes to green, and finally to yellow. This same reaction occurs if the sodium salt of the acetyl is dissolved in distilled water and allowed to stand without the addition of sodium hydroxide. Also if the disodium salt is filtered on a small Buchner funnel, washed with sodium chloride, and allowed to stand in a moist condition there is rapid liberation of iodine and production of bluish green colors, which fade to yellow. If small pieces of the acetyl in dry form are added to water, alcohol, or pyridine containing alkali, the solution of the solid material is accompanied by decomposition in part with a liberation of iodine and discoloration. If the acetyl is dissolved in alcohol the addition of sodium hydroxide to the solution will form the sodium salt of the acetyl without decomposition or liberation of iodine. Furthermore, an alkaline solution can be added to acid with precipi-

tation of the acetyl without decomposition. In both acid and alkaline solutions the acetyl is as stable as thyroxin, but at the neutral point a spontaneous decomposition occurs. One of the factors which affects this reaction is the mass of material present. In dilute solutions the decomposition of the acetyl is very much slower, and in sufficiently dilute solutions it may not occur at all. This effect of the mass of material explains why solution in alcohol prevents the destruction of the acetyl with addition of alkali. The mechanism is essentially the diminution of the concentration of the acetyl. Great difficulty was encountered in the preparation of the acetyl until the factors influencing the decomposition were discovered. As thyroxin does not react in this way no difficulty was anticipated, and it was only after identification of free iodine in the solutions of the acetyl which had turned bluish green that an insight into the mechanism was obtained. Why the acetyl derivative spontaneously decomposes at the neutral point and gives off iodine in the free form instead of hypoiodous acid, as occurs with thyroxin, is not known.

The reactions resulting in the oxidation of the acetyl and liberation of iodine are also given by the ureide under similar conditions. This excludes the possibility that the acetyl radical is necessary for the decomposition and suggests that the reason for the instability is the replacing of the imino hydrogen by a larger group.

When this decomposition has occurred in part, the products cannot be removed from the rest of the material, and it is impossible to separate the acetyl in free form. The tarry products resulting from the decomposition of a small part prevent the crystallization of the rest of the acetyl. The retention of impurities by the acetyl is very similar to the retaining of impurities by partially purified thyroxin.

Beside the spontaneous decomposition, other reactions which are specific to the acetyl were found which will be discussed at this time. It was found that when the acetyl was freshly precipitated from an alkaline solution by an acid it is soluble in ether. After it has been separated in crystalline form and dried it is insoluble in ether. This difference in solubility is undoubtedly due to the acetyl existing in open-ring form when precipitated from cold water solution. The closed-ring form is insoluble in ether.

When the acetyl derivative is prepared by adding acetic anhydride to an alkaline alcoholic solution of thyroxin, and the alcoholic solution of the acetyl is then added to ether the acetyl is removed only partially by subsequent extraction of the ether with sodium hydroxide. A large amount of the acetyl remains in the ether. If the ether solution is tested with nitrous acid the usual reaction with the production of a yellow color turning to red with the addition of ammonia, does not occur. After alcoholic sodium hydroxide is added and the solution is heated, a typical reaction with nitrous acid will take place. Another difference of the acetyl before and after the treatment of the ether solution with alkali is shown in the solubility of the acetyl in alkalies. If the ether is evaporated before alkaline hydrolysis, the acetyl is found to be very difficultly soluble in aqueous sodium hydroxide. After hydrolysis in alkaline alcohol, the acetyl is very easily soluble in dilute alkali. The non-reactivity with nitrous acid and insolubility in alkali may be due either to the formation of an inner salt between the carboxyl and imino, or to the formation of a diacetyl derivative. Treatment with alkalies hydrolyzes either the acid salt or one acetyl group, and the acetyl in free form is liberated.

The Action of Sunlight on Thyroxin, and the Production of Colored Compounds from Thyroxin.

When thyroxin, the acetyl, or the ureide is dissolved in dilute sodium hydroxide and allowed to stand in the sunlight, the solution slowly changes, and in the course of 12 to 72 hours develops a distinctly pink color. On further standing the pink color is changed to yellow. When the carbonic acid derivative was prepared by treating thyroxin with phosgene, it was found to be unstable and changed to a deep pink. When the sodium or barium salt of thyroxin is allowed to stand exposed to sunlight in a dry form, it also develops a pink color. With the barium salt this action does not occur in the dark, or when the salt is covered with water. The development of the pink color in each case is accompanied by the splitting off of iodine in the form of hypoiodous acid. This pink color was first noticed on the edges and outside of white porcelain casseroles which were used to extract

the barium salt of thyroxin with sodium hydroxide. Where the solution dried and was exposed to heat and light the pink color developed. Later this was shown to be due to the thyroxin itself and not to the impurities present. The chemical structure of the pink-colored compound is still unknown, but it appears probable that it is an oxidation product of the hydroindole nucleus. The effect of light on the separation of thyroxin is of importance, and loss of thyroxin due to the action of light may amount to a considerable percentage of the total unless precautions are observed not to permit the action of direct sunlight to destroy the partially purified thyroxin.

Effect of Acid and Alkali on Thyroxin.

No quantitative determinations have been made as yet concerning the ultimate products of alkaline hydrolysis because of the amount required to isolate the decomposition products. Thyroxin is not affected at room temperature by any concentration of aqueous sodium hydroxide. It is soluble in dilute alkali and after the concentration has reached 10 to 15 per cent the disodium salt separates. The further addition of alkali renders the sodium salt more insoluble but it does not cause any destruction of thyroxin. Although thyroxin is stable in sodium hydroxide at room temperature, when it is heated above 110° in the presence of strong sodium hydroxide, there is a destruction of the molecule with the splitting off of sodium iodide, and eventually the liberation of indole which may be identified by the pine-splinter reaction. The amount of indole liberated is not quantitative and it is probable that only traces of the hydroindole nucleus appear as indole.

In acids thyroxin is not so stable as in the presence of alkali. In aqueous solutions of pure thyroxin, hydrochloric or sulfuric acids precipitate the hydrochloride or sulfate of thyroxin, and, since these are insoluble, the destruction of thyroxin is prevented. However, when thyroxin is present in open-ring form, strong acids bring about reactions with the impurities and oily tarry products result. In alcohol solutions prolonged action of hydrochloric acid causes a destruction in part even with pure thyroxin, resulting in the production of a brown discoloration. Poly-

merization of indole compounds in the presence of acid is well established, and it seems probable that this explains the destructive action of acids on thyroxin.

A Consideration of Other Possible Structural Formulas.

Accepting the empirical formula as $C_{11}H_{10}O_3NI_3$, the carbonyl group in the molecule could be present as a ketone, either attached to the side chain or to the benzene ring. That this is not the case is shown by the failure of thyroxin to react with hydrazine, phenylhydrazine, or semicarbazone. The carbonyl group adjacent to the imino should not react with hydrazine and the failure of thyroxin to react is evidence corroborating the hypothesis that the carbonyl group is adjacent to the imino. The positions of the three iodine atoms, the three extra hydrogen atoms, and the three carbon atoms in excess of the indole nucleus have not been determined by substitution or by decomposition products. The most conclusive proof of the position of the three carbon atoms and the terminal carboxyl would be furnished by the synthesis of thyroxin. The synthesis of thyroxin will be reported in another paper, but at this time the synthesis of the compound will be cited as evidence for the correctness of the structural formula assigned in regard to the position of the three carbon atoms with terminal carboxyl. The establishment of the fact that thyroxin does not rotate polarized light excludes an asymmetric carbon atom and confirms the arrangement of the double-bonds.

The Crystal Form and Melting Point of Thyroxin.

The keto form of thyroxin crystallizes in six distinctly different forms (Figs. 25 to 30). Each of the seven other forms of thyroxin has characteristic crystal forms. The di-basic and mono-basic metal salts and the amino- and imino acid salts of thyroxin also have characteristic forms.

The crystal forms of imino acid salts and of di- and mono-metal salts are flat plates for the most part. The other crystal forms are long branching needle blades or thread-like needles which occur in rosettes, or in sheaves, or in tangled masses.

Beside the type of crystal, all these forms and derivatives of thyroxin also have characteristic melting points. For these reasons microscopic study of the crystals and the determination of the melting point are the two methods which have proved of greatest value during this investigation. The determination of iodine is not of such value because the iodine content of any one

FIG. 25.

FIGS. 25 to 30. Six different types of crystals in which the keto form of thyroxin separates.

form may vary within wide limits without greatly affecting either the melting point or the crystal form. A good illustration of this is in the keto form of thyroxin. The keto form of thyroxin when pure melts at 250°, when slightly impure the melting point drops to 246° or 245°, and when grossly contaminated with impurities it drops to 240°. This point, however, appears to be the limiting value below which the keto form seldom melts. It appears that

merely dissolving thyroxin in alkali and precipitating it causes a slight decomposition. The decomposition products are retained by the pure thyroxin and are separated with great difficulty. However, the presence of these impurities rarely exceeds the amount which lowers the melting point to the neighborhood of 240°. Microscopically the keto type of crystals could be identi-

Fig. 26.

fied, and the melting point of 240° or above would confirm the form in which thyroxin existed but the iodine content might vary as much as 2.5 per cent from the theoretical. The keto form melts the highest of all forms or derivatives of thyroxin, from 240–250°, the amino carboxyl form melts in the neighborhood of 225°; the amino hydrate melts at 216°, and the enol form at 204°; the imino acid salts melt at about 228°; the amino-acid

salts in the neighborhood of 204°; the derivatives of thyroxin attached to the imino, in closed-ring form, melt at about 238° and in open-ring form at about 152°. It is apparent that the melting points of different forms and derivatives are so widely separated that no misinterpretation could result except in cases of mixtures, and the crystal form is so definite that mixtures can

FIG. 27.

be identified under the microscope. It has been found, however, that the melting point varies greatly with the rate of heating. One sample of thyroxin, which melted at 240° when heated at the rate of 10° increase per minute, melted at 248° when heated at the rate of 18° increase per minute, and at 221° when heated at the rate of 0.6° increase per minute.

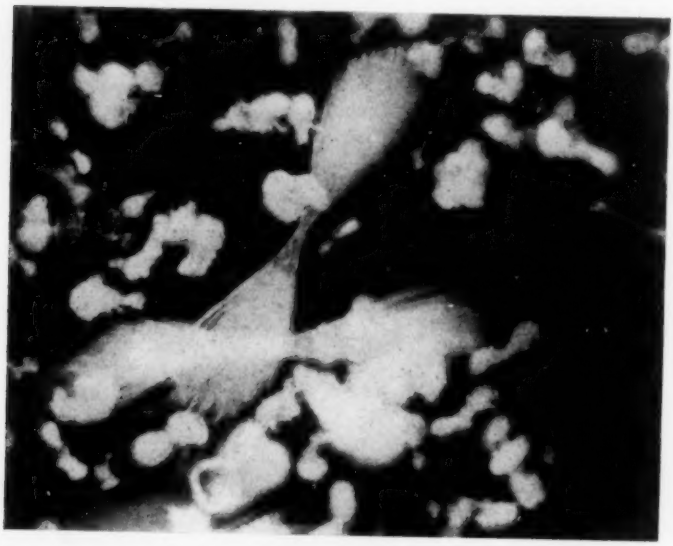


FIG. 28.

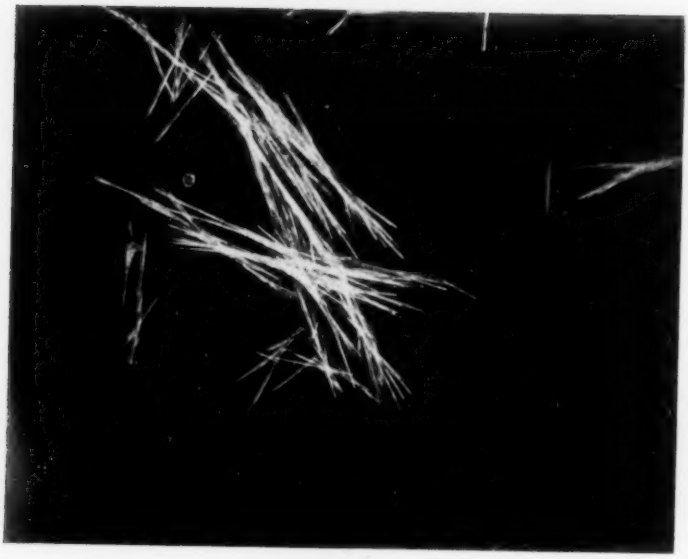


FIG. 29.

For a routine determination we have adopted the rate of 10° increase per minute, and when the melting point is observed under these conditions each form of thyroxin and its derivatives agree very closely in their melting points, with other samples of the same form.

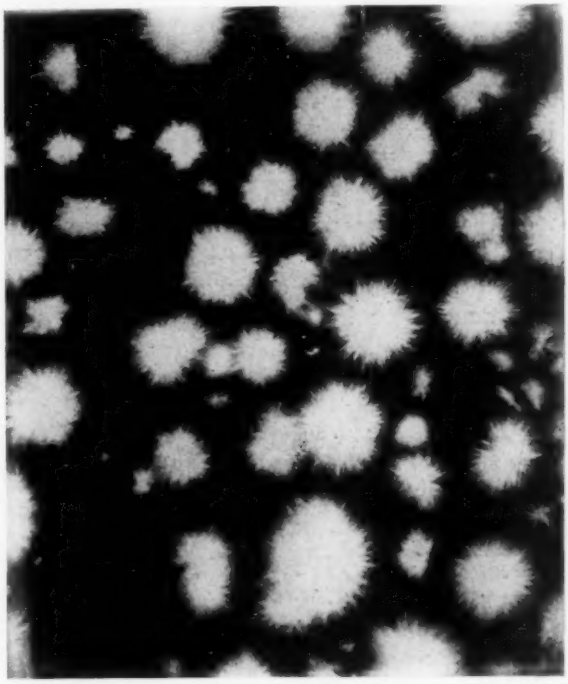


FIG. 30.

EXPERIMENTAL.

The Percentage of Iodine in Thyroxin.

Eighteen different samples of thyroxin have been prepared, the weights of the samples ranging from 900 mg. to 5 gm. All the samples contained at least 63.5 per cent of iodine and six

were purified until they contained approximately 65 per cent of iodine. The iodine content[2] of Samples 1 to 6 is as follows:

Iodine Content of Thyroxin.[2]

Sample 1.	5.10 mg.	contained	3.31 mg.	of	iodine	=	64.81	per cent.
" 2.	5.12 "	"	3.32 "	"	"	=	64.86	" "
" 3.	5.24 "	"	3.41 "	"	"	=	65.02	" "
" 4.	5.10 "	"	3.32 "	"	"	=	65.00	" "
" 5.	25.3 "	"	16.45 "	"	"	=	65.00	" "
" 6.	4.51 "	"	2.66 "	"	"	=	65.02	" "

Precipitation of thyroxin from alkaline alcohol with acetic acid, or by boiling an ammoniacal solution, does not vary the percentage of iodine. Precipitated with acetic acid from alkaline alcohol the iodine content of thyroxin was 64.95 per cent. Precipitation of the same preparation by boiling an ammoniacal solution gave an iodine content of 64.92 per cent. 50 mg. of thyroxin were dissolved in 200 cc. of water containing 3 cc. of concentrated ammonium hydroxide. The solution was boiled down to 100 cc. 100 cc. of water were added and the solution was boiled for a few minutes, cooled, and filtered. 15 cc. of filtrate contained 0.20 mg. of iodine.

Solubility = one part in 48,800.

Ultimate Analysis of Thyroxin.

Sample No.	Weight of sample.	CO_2	H_2O	Carbon.	Hydrogen.	Iodine
	mg.	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*
7	201.4	175.2	32.3	23.72	1.78	64.95
7	171.2	148.0	26.9	23.57	1.74	64.95
8	291.0	248.8	45.7	23.32	1.74	64.92
9	124.2	101.9	18.5	22.37	1.65	65.02

102 mg. of thyroxin contained 2.27 mg. of nitrogen = 2.23 per cent.

	Carbon.	Hydrogen.	Oxygen.	Nitrogen.	Iodine.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Calculated for $C_{11}H_{10}O_3NI_3$	22.56	1.70	8.20	2.39	65.10
Found	22.37	1.65	8.73	2.23	65.02

[2] The iodine was determined by a method published in 1914, which has been modified recently so that it is now applicable for the determination of small amounts of iodine to a high degree of accuracy (The determination of iodine in connection with studies in thyroid activity, Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251).

Solubility of Keto Form of Thyroxin.

50 mg. of thyroxin were dissolved in 200 cc. of dilute sodium hydroxide. 20 cc. of 50 per cent hydrochloric acid were added and the solution was boiled; 15 cc. of the filtrate contained 0.116 mg. of iodine.

Solubility = one part in 84,000.

The Sulfate of Thyroxin.—For the preparation of the sulfate 50 mg. of thyroxin are dissolved in 200 cc. of water containing 50 to 100 mg. of sodium hydroxide; 20 cc. of 50 per cent sulfuric acid are added, and the solution is boiled. The sulfate of thyroxin is soluble at 100°, but on cooling settles to the bottom of the container in oval-shaped plates. It may be filtered on a Buchner funnel, washed with water, and dried in a desiccator.

Analysis of the Sulfate of Thyroxin.

Sample 1. 5.48 mg. contained 3.27 mg. of iodine = 59.71 per cent.
" 2. 25.00 " " 15.00 " " " = 60.00 " "

114.4 mg. of thyroxin sulfate gave 86.9 mg. of carbon dioxide, 18.1 mg. of water; 5.08 mg. contained 3.04 mg. of iodine.

	Carbon.	Hydrogen.	Iodine.
	per cent	*per cent*	*per cent*
Calculated for $C_{11}H_{11}O_5NI_3S$	20.82	1.73	60.09
Found	20.71	1.75	59.92

50 mg. of thyroxin were dissolved in 200 cc. of sodium hydroxide precipitated with 20 cc. of 50 per cent sulfuric acid, and the solution was boiled; 15 cc. of the cooled filtrate contained 0.127 mg. of iodine.

Solubility = one part in 76,900.

The Hydrochloride of Thyroxin.—The hydrochloride of thyroxin is best prepared by dissolving a small amount of thyroxin, 15 to 25 mg. in 5 or 6 cc. of alcohol containing 2 to 3 cc. of 50 per cent hydrochloric acid. 1 to 2 cc. of water are added, and the alcohol is boiled off in a test-tube. After most of the alcohol has been distilled the hydrochloride will separate in flat, glistening plates. If too small an amount of acid is used, or too much water, the hydrochloride will hydrolyze, and the plates will change to needles.

The Metal Salts of Thyroxin.—Dry crystalline thyroxin is readily soluble in sodium and potassium hydroxides in the cold, if the concentration of the alkali is less than 10 to 15 per cent. Thyroxin is insoluble in sodium or potassium hydroxide in the cold if the alkali is stronger than 15 to 20 per cent, but it is more soluble in strong alkali if the solution is warmed. In a mixture of 66 per cent of alcohol and 33 per cent of water containing 1 per cent of sodium hydroxide, thyroxin may be dissolved to the extent of about 4 per cent. It is still more soluble in hot solutions but on cooling will separate as the di-metal salt. Thyroxin is not readily soluble in dilute ammonia, but concentrated ammonia will dissolve about 1.8 per cent of thyroxin.

The Di-Basic Sodium and Potassium Salts.—100 mg. of thyroxin are dissolved in 20 cc. of dilute sodium hydroxide and to this are added 150 cc. of a solution of 10 per cent sodium hydroxide containing 20 per cent of sodium chloride. If a precipitate occurs the solution is warmed and the clear solution is then allowed to stand until cold. The exact concentrations of sodium chloride and hydroxide are not important. The disodium salt readily separates in any solution containing a high concentration of sodium salts. If the strongly alkaline solution is decanted and replaced with 15 per cent sodium chloride solution, the crystals may be filtered through a Buchner funnel on paper. They are insoluble in 10 to 15 per cent sodium chloride and may be washed and dried. The disodium salt cannot be prepared in pure form for analysis as washing with water dissolves the salt, and it passes through the paper. The di-basic potassium salt is prepared in a way similar to that used for the sodium salt. The diammonium salt is prepared by dissolving thyroxin in hot concentrated ammonium hydroxide and allowing the solution to cool. Flat, rectangular crystals of the diammonium salt will separate.

The Alkaline Earth Salts of Thyroxin.—The barium, calcium, and magnesium salts of thyroxin are prepared by dissolving 50 to 100 mg. of thyroxin in 100 cc. of dilute sodium hydroxide using as small an amount of alkali as possible to carry the thyroxin into solution. The solution is heated to boiling and 20 cc. of a 20 per cent solution of barium, calcium, or magnesium chloride are added to the hot solution of thyroxin. Carbon dioxide is excluded by placing the beaker in an atmosphere free of carbon

dioxide. The magnesium and calcium salts are very slightly soluble in boiling water. 500 cc. of boiling water will dissolve between 100 and 150 mg. of the barium salt. The barium salt may be suspended in boiling water and the solution filtered through a Buchner funnel into a suction flask. 30 cc. of 20 per cent barium chloride are now added to the filtrate and the barium salt is allowed to recrystallize. Only a negligible amount of thyroxin is soluble in the presence of this amount of barium chloride. The barium salt under these conditions is unstable and will slowly turn a pink color. Analysis for iodine shows a lower iodine content than that calculated.

Iodine Content of Barium Salt.

Sample 1.	6.76 mg.	contained	3.26 mg.	of iodine =	48.51 per cent.
" 2.	5.98 "	"	3.00 "	" " =	50.24 " "
Calculated for $BaC_{11}H_8O_3NI_2$:					52.91 " "

If the barium salt is filtered and dried there is a slow decomposition and the color of the salt becomes yellowish gray.

Silver, Copper, Nickel, and Zinc Salts of Thyroxin.—The silver, copper, nickel, and zinc salts are prepared by dissolving 50 to 100 mg. of thyroxin in 25 to 50 cc. of concentrated ammonia. 25 cc. of a 10 per cent solution of silver nitrate, copper sulfate, nickel sulfate, or zinc sulfate are made ammoniacal with strong ammonia so that the precipitated hydroxide is just carried into solution, and there is a slight excess of ammonia. The solution of the metal is now added to the ammoniacal solution of thyroxin, and after standing a short time the crystals of the metal salt will separate. If too much ammonia is used, a larger amount of the solution of the metal may be required to start the crystallization. Allowing it to stand over night will insure a more complete precipitation. Sodium hydroxide may be used for the solution of thyroxin, but, since the metal salt of thyroxin is more soluble in sodium hydroxide, ammonium hydroxide has been found to be the most satisfactory solution. The silver, copper, nickel, or zinc salts suspended in water or dilute ammonia are soluble on the addition of several cc. of 30 per cent sodium hydroxide solution.

Iodine Content of Silver Salt.

Sample 1.	4.86	mg.	contained	2.37	mg.	of	iodine	= 48.74	per cent.
" 2.	4.96	"	"	2.44	"	"	"	= 49.15	" "
" 3.	4.00	"	"	1.97	"	"	"	= 49.34	" "
" 4.	4.06	"	"	1.99	"	"	"	= 49.10	" "
" 5.	4.54	"	"	2.23	"	"	"	= 49.20	" "
" 6.	4.08	"	"	1.96	"	"	"	= 48.00	" "
" 7.	6.02	"	"	2.93	"	"	"	= 48.60	" "
" 8.	4.38	"	"	2.09	"	"	"	= 47.73	" "
Calculated for $Ag_2C_{11}H_8O_3NI_3$:								47.74	" "
" " $AgC_{11}H_9O_3NI_3$:								55.06	" "

The average of these eight determinations is 48.73 per cent iodine. The iodine content indicates the addition of 92 per cent of the theoretical amount for a di-metal salt and 184 per cent of the amount required for a mono-metal salt. The higher iodine content than that calculated for a di-metal salt is undoubtedly due to the hydrolysis of the hydroxy group. In the zinc salt, the amount of iodine was found to be 56.95 per cent. The calculated amount for the zinc salt is 58.79 per cent, but the calculated amount of iodine in the zinc salt in which hydrolysis of the hydroxy group had occurred would be 57.12 per cent. The close agreement between the amount found and the latter figure is evidence that hydrolysis of the hydroxy group also occurs in the zinc salt of thyroxin. The low iodine content of the salts of the diad metals, barium and zinc, and the high iodine content of the salt of the monad metal, silver, suggest that in all di-metal salts of thyroxin hydrolysis of the hydroxy group is brought about by washing the salt with water.

Preparation of Mono-Metal Salts.—100 mg. of thyroxin are dissolved in 150 cc. of 1 per cent sodium hydroxide, and carbon dioxide is bubbled through the solution until thyroxin is precipitated. The suspension is now heated until solution is complete. On cooling, the monosodium salt separates in crystal form. The separation of the mono-salt is assisted by the presence of sodium salts and is hindered by too great a dilution. An excess of carbon dioxide must not be passed through the solution, as free thyroxin will be precipitated. The preparation of the potassium salt is similar to that of the sodium salt. In preparing the ammonium salt, strong ammonium hydroxide is much better than dilute, as

the solubility of the ammonium salt is thereby decreased. If the mono-metal salt is filtered on a Buchner funnel and washed with 10 to 15 per cent sodium, ammonium, or potassium chloride, it is not dissolved through the paper. If washed with cold water, about 40 per cent is dissolved and 60 per cent of the amount of thyroxin taken remains on the paper in the form of free thyroxin in the enol form.

Nesslerization of the Hydrolyzed Monoammonium Salt of Thyroxin.

50 mg. of the residue left on the paper after washing the monoammonium salt with water were dissolved in 60 cc. of ammonia-free water containing a small amount of sodium hydroxide. The addition of 15 cc. of Nessler's solution and dilution to 100 cc. produced no color, showing hydrolysis of the monoammonium salt was complete.

Solubility of Monosodium Salt.—5 cc. of 0.10 per cent sodium carbonate dissolved about 15 mg. of dry thyroxin in keto form, when the solution was heated to boiling. Most of the thyroxin reprecipitated on cooling to 20°, but not as the monosodium salt. The crystals were needles or very small rosettes. 1 cc. of the filtrate contained 0.76 mg. of iodine = 1.17 mg. of thyroxin.

Solubility = one part in 850.

5 cc. of 1 per cent sodium carbonate dissolved 85 to 90 mg. of thyroxin at almost the boiling point. Probably more than this amount could be dissolved but as the solution began to turn yellow the heating was stopped. On cooling to 23°, needles in clusters and square plates of monosodium salt separated. 1 cc. of filtrate contained 1.14 mg. of iodine = 1.75 mg. of thyroxin.

Solubility = one part in 570.

The Enol Form of Thyroxin.—The enol form of thyroxin is the most difficult to prepare in crystalline form, but is very easily prepared in the crystal form of the mono-metal salts by cold water hydrolysis of the monoammonium, sodium, or potassium salts. 10 to 20 mg. of the enol form in dry powder is readily soluble in 1 to 2 cc. of pyridine. The addition of 10 to 15 cc. of water will produce a cloudiness. On long standing (24 hours), the thyroxin will precipitate. Depending on conditions of the concentration of pyridine and the amount of thyroxin present,

the thyroxin may precipitate in keto form. This may be distinguished under the microscope as long bundles, or sheaves of needles. The enol form separates in either rosettes, or short bundles of needles. The enol form of thyroxin is also soluble in quinoline. Other ways to prepare the enol form of thyroxin is to add ammonium chloride to a cold solution of the disodium salt in water which does not contain an excess of alkali, or to dissolve the disodium salt of thyroxin in alcohol and add water and ammonium chloride. A mixture of both keto and enol crystals usually results if much alcohol is present.

10 to 15 mg. of dry crystals of the keto form of thyroxin may be boiled with 5 to 10 cc. of pyridine without going into solution. The addition of a very small amount of dilute ammonia will change the thyroxin to the enol form and carry it into solution. The water may then be boiled off and the enol form remains soluble in pyridine. If 15 to 20 mg. of the enol form of thyroxin are dissolved in 2 to 3 cc. of pyridine in a test-tube, and to this 10 to 15 cc. of water are added, and the solution is boiled, after the pyridine has been removed by distillation, thyroxin will separate in the keto form similar to the precipitation of thyroxin from a boiling ammoniacal solution.

25 mg. of thyroxin added in the keto form to 10 cc. of water in the presence of 1 gm. of sodium carbonate will not dissolve. If alcohol is used instead of water and the alcohol is boiled, the sodium carbonate does not dissolve but the thyroxin is carried into solution. The monosodium salt of thyroxin in the enol form produced by the alcoholic suspension of sodium carbonate is readily soluble in alcohol.

The Amino-Acid Salt Form of Thyroxin.—For the preparation of amino-acid salts, thyroxin is dissolved in a small amount of sodium hydroxide in a large volume of water, and to this carbon dioxide or other organic acid is added until thyroxin precipitates. The voluminous precipitate is filtered, washed, and dried. In order to prepare the amino salts in crystalline form, other expedients may be used. For the preparation of the amino sulfate, thyroxin is dissolved either in formic acid or in alcohol containing a small amount of sulfuric acid. The alcohol is concentrated to small volume, and water is quickly added in large volume.

Solubility of Thyroxin in Amino Salt Form.

Hydrochloride.—50 mg. of thyroxin were dissolved in 200 cc. of dilute sodium hydroxide, and precipitated at 25°C. with a slight excess of hydrochloric acid, 15 cc. of the filtrate, after removing the precipitate of thyroxin, contained 0.037 mg. of iodine.

Solubility = one part in 263,000.

Carbonate.—50 mg. of thyroxin were dissolved in 200 cc. of dilute sodium hydroxide, and precipitated by passing carbon dioxide through the solution; 15 cc. of the filtrate contained 0.012 mg. of iodine.

Solubility = one part in 815,000.

Iodine Content of Amino Carbonate.

5.8 mg. contained 3.137 mg. of iodine = 60.57 per cent.
Calculated for $C_{11}H_{12}O_4NI_3(H_2CO_3)_{\frac{1}{2}}$ = 60.09 " "

The slightly higher figure for iodine than the calculated amount is probably due to separation of the thyroxin in either the enol or amino hydrate form, or to the hydrolysis of the amino carbonate and formation of the amino carboxyl. This sample of thyroxin contained 65.02 per cent of iodine when precipitated in keto form.

The Amino Carboxyl Form of Thyroxin.—The amino carboxyl form is prepared by suspending the amino-acid salt form of thyroxin in water in the presence of a small amount of weak organic acid, such as acetic, and boiling the solution. The acid radical is expelled from the amino group, and the adjacent carboxyl group forms an amino carboxyl salt. It can also be prepared by dissolving the disodium salt of thyroxin in a large volume of water, heating to boiling, adding ammonium chloride, and continuing the boiling. The amino carboxyl form of thyroxin separates.

If no acid is present the elements of water may be expelled from the molecule and thyroxin will separate in keto form.

Iodine Content of Hydrolyzed Amino-Acid Salts.

A sample of amino carbonate was suspended in neutral distilled water and the solution was boiled 3 minutes. This was not sufficiently long to complete the hydrolysis.

5.38 mg. contained 3.321 mg. of iodine = 61.72 per cent.

The same sample boiled 15 minutes showed that all of the carbonate had been expelled and also that some of the amino carboxyl form had been converted into the keto form.

5.2 mg. contained 3.305 mg. of iodine = 63.56 per cent.

A sample of amino sulfate suspended in distilled water and boiled more nearly approximated the amino carboxyl form, but in this case all the sulfate radical was not hydrolyzed.

5.0 mg. contained 3.135 mg. of iodine = 62.72 per cent.
Calculated for amino carbonate, 60.09 per cent iodine.
" " " sulfate, 58.43 " " "
" " " carboxyl, 63.18 " " "

Preparation of the Amino Hydrate Form.—Thyroxin is dissolved in a large volume of water with a moderate excess of sodium hydroxide. The solution is heated to boiling and then removed from the flame, and 10 per cent ammonium chloride is slowly added to the amount of 10 to 15 cc. The solution becomes turbid, and long branching crystals separate. The limits of the concentration of the hydrogen ion and the temperature for the formation of the enol, amino hydrate, amino carboxyl, and the keto forms are very narrow. If thyroxin is dissolved in a few mg. of sodium hydroxide, and the solution is diluted to about 400 cc., and divided into four equal parts, each of the four different forms of thyroxin may be prepared from these solutions merely by varying the conditions of the precipitation. Ammonium chloride added to one of the solutions in the cold, will precipitate the thyroxin in the enol form, or as the monoammonium salt in flat plates. If ammonium chloride is added to the second solution, which has been heated to boiling, and then removed from the flame, the amino hydrate form will separate. If the amino hydrate is precipitated in the third, and the solution containing a suspension of the amino hydrate is heated to boiling and the boiling continued, the crystals will change into the amino carboxyl form. If a large excess of ammonium chloride is added to the fourth solution, and the solution is boiled, thyroxin will separate in the keto form.

Open-Ring Form in the Presence of Impurities.—The percentage of iodine in the open-ring form of thyroxin, which is still soluble

in pyridine, sodium carbonate, barium hydroxide, and alcohol, may be between 50 and 60 per cent.

Iodine Content of Thyroxin Still in Open-Ring Form.

Sample 1.	5.00	mg.	contained	2.51	mg.	of	iodine	= 50.28	per	cent.
" 2.	3.92	"	"	2.13	"	"	"	= 54.21	"	"
" 3.	6.44	"	"	3.51	"	"	"	= 54.46	"	"
" 4.	4.16	"	"	2.43	"	"	"	= 58.53	"	"

The solubility of these samples in alcohol, sodium carbonate, and barium hydroxide showed that although the amount of impurities present was very small, the ring still existed in open form.

Colloidal Substances Producing the Open-Ring Form of Thyroxin.—50 to 100 mg. of pure thyroxin, added to the impurities which are separated during the process of purification, will become readily soluble in alcohol, sodium carbonate, and barium hydroxide, showing the change from the keto to the open-ring form. Gelatin and proteins of blood produce the same changes, but the amino-acids resulting from the hydrolysis of gelatin will not change the solubility of thyroxin in barium hydroxide, alcohol, or sodium carbonate.

The Acetyl Derivative.

Preparation of the Acetyl.—In the preparation of the acetyl it is necessary to use pure thyroxin. The presence of even a small amount of impurities makes it impossible to crystallize the acetyl and it will separate only as an oily tar. 100 mg. of pure thyroxin are added to 20 cc. of alcohol containing 100 mg. of sodium hydroxide. After the thyroxin is entirely dissolved 2 cc. of acetic anhydride are added. The solution is allowed to stand for 30 minutes, 5 cc. of water and 5 cc. of 50 per cent sulfuric acid are added, and the alcohol is evaporated by boiling in a 200 cc. distilling flask under diminished pressure. The temperature is not allowed to go above 40°C. Crystals of the sulfate of the acetyl separate as the alcohol is removed. These are dissolved in about 15 cc. of alcohol which is filtered and added to a beaker containing 200 cc. of boiling water and 5 cc. of 50 per cent sulfuric acid. The addition of the first few drops of the alcohol solution of the acetyl does not cause a precipitate but further addition of the alcohol solution causes a precipitation in crystalline form of the free acetyl.

Purification of the acetyl may also be carried out by dissolving the acetyl sulfate in 25 cc. of alcohol and adding 5 gm. of sodium acetate and 10 cc. of 30 per cent sodium hydroxide. After the alcohol has been removed by boiling under diminished pressure, the disodium salt of the acetyl will separate in large, flat plates. The sodium salt may then be dissolved in alcohol and precipitated by addition to a boiling solution of dilute sulfuric acid as described above.

Many samples of the acetyl have been prepared and analyzed. Some of the results are as follows:

Analysis of the Acetyl of Thyroxin.

Sample	1.	5.26	mg.	contained	3.20	mg.	of	iodine	= 60.91	per cent.
"	2.	5.20	"	"	3.17	"	"	"	= 60.96	" "
"	3.	3.12	"	"	1.90	"	"	"	= 60.85	" "
"	4.	5.12	"	"	3.11	"	"	"	= 60.67	" "
"	5.	5.16	"	"	3.14	"	"	"	= 60.83	" "
"	6.	5.10	"	"	3.11	"	"	"	= 60.93	" "
"	7.	5.10	"	"	3.09	"	"	"	= 60.61	" "
"	8.	5.04	"	"	3.05	"	"	"	= 60.52	" "
"	9.	5.02	"	"	3.05	"	"	"	= 60.74	" "

97.9 mg. of acetyl gave 90.1 mg. of carbon dioxide and 16.2 mg. of water; 5.10 mg. contained 3.10 mg. of iodine.

	Carbon.	Hydrogen.	Iodine.
	per cent	*per cent*	*per cent*
Calculated for $C_{13}H_{12}O_4NI_3$	24.88	1.93	60.77
Found	25.09	1.85	60.86

The Sulfate of the Acetyl Derivative.—If the acetyl derivative is dissolved in alcohol and added to boiling dilute sulfuric acid, the acetyl precipitates in free form without the addition of sulfuric acid attached to the imino group. If, however, sulfuric acid is added to the alcohol solution of the acetyl and the alcohol is evaporated either at room temperature by a current of air or by boiling under diminished pressure at a low temperature, the sulfate of the acetyl separates in needle crystals.

Analysis of the Sulfate of the Acetyl.

Sample	1.	5.02	mg.	contained	2.84	mg.	of	iodine	= 56.66	per cent.
"	2.	5.52	"	"	3.12	"	"	"	= 56.52	" "
"	3.	6.2	"	"	3.51	"	"	"	= 56.61	" "

111.5 mg. of the sulfate of the acetyl gave 94.8 mg. of carbon dioxide and 19.7 mg. of water; 5.52 mg. contained 3.12 mg. of iodine.

	Carbon.	Hydrogen.	Iodine.
	per cent	*per cent*	*per cent*
Calculated for $C_{15}H_{12}O_6NI_3S_{\frac{1}{2}}$.....	23.07	1.92	56.36
Found.....	23.18	1.91	56.52

Although thyroxin is insoluble in pyridine, alcohol, ethyl acetate, or acetic acid, the acetyl is soluble in all these reagents. When the acetyl is precipitated from an alkaline solution by the addition of an acid, it is at first soluble in ether and may be extracted out of the water by placing in a separatory funnel with ether. After the acetyl has been prepared in pure form and dried, it is insoluble in ether.

The Di-Metal Derivatives of the Acetyl.[3]—The sodium, ammonium, and potassium salts of the acetyl are prepared as with thyroxin. 100 mg. of the acetyl are dissolved in 10 cc. of alcohol, to which are added 10 cc. of 30 per cent sodium hydroxide and 5 gm. of sodium acetate. The di-metal salt will separate in large, flat crystals if the alcohol is evaporated under diminished pressure. The temperature may be raised to that of boiling water without decomposition of the acetyl by the sodium hydroxide as no hydrolysis of the acetyl occurs under these conditions. If sodium chloride is substituted for sodium acetate in a solution similar to the one mentioned above and the alcohol is evaporated, the sodium salt of thyroxin separates in small plates.

The barium and calcium salts of the acetyl are prepared by dissolving 25 to 50 mg. of the acetyl in the least possible amount of alcohol, 2 to 3 cc., adding water and ½ to 1 cc. of pyridine; the solution is boiled until the alcohol is volatilized, care being taken not to decompose the acetyl by prolonged boiling. To the pyridine solution of the acetyl a 40 per cent solution of barium or calcium chloride is slowly added, and the solution is heated to boiling. The alkaline earth salt of the acetyl will separate. It is readily soluble in an excess of pyridine. If the solution is heated for too long a time hydrolysis will occur. The salt may be filtered and washed without decomposition.

[3] In working with the acetyl, it is always necessary to dissolve the dry powder in alcohol before making it alkaline. Unless this is done, some of the acetyl will spontaneously decompose during the solution of the powder in alkali.

The reactions of thyroxin indicate that the molecule exists in both open- and closed-ring forms. The chemical properties of the acetyl are evidence that this derivative also exists in open- and closed-ring forms. The sodium salt of the acetyl washed with dilute acetic acid is completely hydrolyzed. When prepared in this way, however, its melting point is found to be 152°. When this material is dissolved in alcohol and added to boiling dilute sulfuric acid, the acetyl separates in closed-ring form, and the melting point is 238°. This suggests that in the sodium salt the acetyl exists in open-ring form, and hydrolysis of the sodium from the molecule leaves the open-ring structure. This is further corroborated by analysis of the silver salt.

Preparation of the Silver Salt of the Acetyl.—If the acetyl is dissolved in alcohol and then made alkaline with sodium hydroxide and the alcohol evaporated, the addition of ammonia and silver nitrate produces no precipitate. This is also true of thyroxin in the open-ring form. If 50 mg. of the acetyl are dissolved in 2 to 3 cc. of alcohol, to which are added 2 to 3 cc. of pyridine and 10 cc. of water, and the alcohol is removed by boiling, the addition of silver nitrate to the aqueous pyridine solution of thyroxin will cause a precipitate to form. This is not crystalline in nature, although under some conditions it may be possible to separate it in crystal form. The silver salt may be filtered on a Buchner funnel and washed with water without decomposition.

5.14 mg. of the silver salt of the acetyl contained 2.26 mg. of iodine = 44.06 per cent.

Calculated for $Ag_2C_{13}H_{12}O_5NI_3$ iodine = 44.35 per cent.
" " $AgC_{13}H_{11}O_4NI_2$ " = 51.91 " "

The iodine content of the silver salt of the acetyl indicates the addition of 2 per cent too much silver for the disilver salt of the open-ring form of the acetyl, and 220 per cent too much silver for the mono-salt of the closed-ring form. These results, proving conclusively that not one but two atoms of silver had added to the acetyl, show that in an alkaline solution the acetyl exists in open-ring form.

The open-ring structure of the acetyl is also indicated by the increased solubility of the acetyl in weak bases such as pyridine, quinoline, and very dilute ammonia. The terminal carboxyl in

thyroxin is so weak that the acetyl derivative would not be soluble in these reagents in the closed-ring form.

Preparation of the Ureide.—Only pure thyroxin should be used in preparation of the ureide for the same reasons given under the preparation of the acetyl. 100 mg. of disodium or zinc salt of thyroxin are added to 10 to 15 cc. of glacial acetic acid containing 200 mg. of potassium cyanate. Solution of the salt of thyroxin should be completed as the ureide is soluble in acetic acid. 5 cc. of water, 5 cc. of alcohol, and 5 cc. of 50 per cent sulfuric acid are added and the alcohol, water, and acetic acid are removed under diminished pressure, the same as in the preparation of the acetyl. The sulfate of the ureide is dissolved in 15 cc. of alcohol, filtered, and slowly added to a beaker containing 200 cc. of water and 5 cc. of 50 per cent sulfuric acid, which is heated to boiling. The ureide separates in crystal rosettes or needles.

Sample 1. 4.78 mg. of ureide contained 2.91 mg. of iodine = 60.81 per cent.
" 2. 5.04 " " " " 3.07 " " " = 61.03 " "
" 3. 5.02 " " " " 3.05 " " " = 60.74 " "
Calculated for $C_{12}H_{11}O_3N_2I_3$ = 60.67 per cent.

Preparation of the Methyl Ester.—100 mg. of the silver salt of thyroxin are suspended in 20 cc. of alcohol to which are added 4 to 5 cc. of methyl iodide. The crystals are occasionally stirred and allowed to stand at a temperature of 40 to 50° for several hours until decomposition of the silver salt is complete. This is indicated by separation of silver iodide in voluminous form. The silver iodide is removed by filtration. If the alcohol is allowed to evaporate, the dimethyl derivative crystallizes in the form of fine threads. They may be separated by formation of the sulfate similar to the preparation of the acetyl. 5 cc. of water and 5 cc. of 50 per cent sulfuric acid are added to the alcohol filtrate from the silver iodide and the alcohol is removed by distillation under diminished pressure. The dimethyl derivative separates probably as the sulfate. It is insoluble in water, but soluble in alcohol. Treatment with alcoholic sodium hydroxide frees the carboxyl group, and the monomethyl derivative is then slightly soluble in aqueous sodium hydroxide. The methyl ester is very difficultly purified and has not been prepared containing a theoretical percentage of iodine. It appears to retain silver iodide even after repeated precipitation.

The Action of Nitrous Acid on Thyroxin.—25 mg. of thyroxin dissolved in 2 cc. of dilute sodium hydroxide were added to the deaminizing chamber of the Van Slyke apparatus, and the usual procedure was followed, shaking for 3 minutes. 0.69 cc. of nitrogen was evolved. The apparatus was allowed to stand 9 minutes and was again shaken for 3 minutes; 0.06 cc. of nitrogen was obtained. 4 minutes later it was again shaken for 3 minutes and 0.04 cc. was obtained. The total, liberated in 22 minutes, showed 1.72 per cent amino nitrogen which is 72 per cent of the total nitrogen contained in the molecule.

Another 25 mg. sample of thyroxin, after 3 minutes shaking, liberated 0.75 cc. of nitrogen. This is equivalent to 1.60 per cent of amino nitrogen in the molecule and is 67 per cent of the total nitrogen.

25 mg. of the amino carboxyl salt form of thyroxin suspended in 2 cc. after 3 minutes shaking gave 0.08 cc. 10 minutes later, after 3 minutes more shaking, 0.06 cc., and 10 minutes later, after 3 minutes shaking, 0.04 cc.; total amount liberated = 0.18 cc. which is equivalent to 0.093 mg. of amino nitrogen, and is 16 per cent of the total nitrogen. 10 mg. of keto form of thyroxin suspended in 2 cc. of water gave no amino nitrogen.

The amino nitrogen in 25 mg. of the acetyl was determined as described above. After 3 minutes 0.11 cc., and after 10½ minutes, 0.11 cc. more of nitrogen were liberated. The nitrogen in the amino form amounted to 24 per cent of the total nitrogen in the molecule.

The nitrogen in isatin and indole, given off as amino nitrogen, was determined. 100 mg. of isatin after 4 minutes gave 1.72 cc. and after 5 minutes more gave 0.41 cc. of nitrogen. This volume was equivalent to 1.13 mg. of nitrogen and amounted to 12 per cent of the total nitrogen present. 100 mg. of indole gave 1.27 cc. after 3 minutes, and 0.34 cc. of nitrogen after 4 minutes more shaking. This is equivalent to 0.86 mg. and is 7 per cent of the total nitrogen in indole.

Besides the action of nitrous acid on thyroxin liberating some of the nitrogen as amino nitrogen, a characteristic color reaction is produced. A few mg. of pure thyroxin, added to 5 cc. of alcohol containing three to four drops of 50 per cent hydrochloric to which are added five or six drops of 1 per cent sodium nitrite solution,

will develop a yellow color. This is increased by boiling. If the solution is cooled and concentrated ammonia is added until distinctly alkaline, a pink color is produced. This is a sensitive reaction for thyroxin, a distinct color being produced by one part of thyroxin in 40,000 parts of solution. If acetic or sulfuric acid is substituted for hydrochloric, the yellow color is not so deep and the addition of ammonia does not produce a pink color but gives a yellowish orange color. If the sample of thyroxin is impure, a yellow instead of a pink color is produced with ammonia. The acetyl and ureide derivatives give the same reaction, producing a pink color with ammonia.

The Pine-Splinter Reaction for Indole.—From 15 to 20 mg. of thyroxin are placed in a test-tube with 5 cc. of 30 per cent sodium hydroxide and sufficient water to carry the thyroxin into solution. As the excess of water is boiled off, thyroxin will precipitate as a disodium salt, but on further heating, after the water has been almost completely driven off and the temperature of the solution has been raised to between 100 and 200°, the disodium salt is again dissolved and a faint indole-like odor is given off. A pine-splinter moistened with hydrochloric acid is turned red by the vapors given off from the fusion.

The Effect of Thyroxin on Polarized Light.—1 gm. of thyroxin was dissolved in 20 cc. of alcohol and 7 cc. of water containing 300 mg. of sodium hydroxide. The solution was filtered and placed in a 2 dm. tube and its effect on polarized light was determined. No rotation of light could be determined. The solution of thyroxin was then placed in a 1 dm. tube. No rotation of the light could be determined.

The Effect of Hydrazine on Thyroxin.—If thyroxin is added to hydrazine hydrate, it immediately dissolves. If water is added, thyroxin remains in solution. The addition of carbon dioxide precipitates thyroxin as the amino carbonate. If the solution of thyroxin in the hydrazine hydrate is boiled after the addition of water, thyroxin precipitates as from ammoniacal solutions. No condensation with hydrazine, phenylhydrazine, semicarbazone, or hydroxylamine could be demonstrated when thyroxin was dissolved in alkaline alcohol to which these reagents were added, in acid alcohol to which these reagents were added, together with sodium carbonate or pyridine, or when dissolved in pyridine to

which the reagents were added. Either decomposed products due to heating at too high temperature or unchanged thyroxin were recovered.

The Melting Points of Thyroxin and Its Derivatives.—The following melting points of thyroxin in its several forms and its derivatives are recorded to illustrate the agreement between different samples (Tables I to IX). It is evident that among some of the derivatives fairly large variations occur, but between any two different forms the differences are much greater than that found between the melting points of two samples of the same derivatives.

The following salts of thryoxin and its derivatives were not melted when heated to 260°: di- and monosodium salt of thyroxin; di- and monopotassium salt of thyroxin; barium salt of thyroxin; disilver salt of thyroxin; disodium salt of acetyl; disilver salt of acetyl; calcium salt of acetyl.

TABLE I.

The Melting Point of the Keto Form of Thyroxin.

No. of sample.	Slight browning.	Sublime or mist.	First droplets.	Completely melted.	Froth.
125			248	250	250
32			241	248	248.9
123			235	247	247
124			240	246	246
81			243	245	246
91			241	242	243
107			241	242	242
115			239	242	242
119			239	241	241.5
117			237	241	241
97			236	240	241
28			195	240	241.5
80			239	240	240
59			238	239	240.5
51			236	239	240

TABLE II.

The Melting Point of the Enol Form of Thyroxin.

No. of sample.	Slight browning.	Sublime or mist.	First droplets.	Completely melted.	Froth.
40	190		200	202	205
41	180		200	205	205
42	190		200	204.5	204.5
43	160	190	200	204	204
44	160	190	202	208	208
46	170	190	200	202	204
49	165	190	203	206	206
50	165		196	201	201
53	150		198	203	204
55	160	190	200	202	204
108	160		198	204	205
109	180	198	206	208	208
110	170		200	202	203
111				203	205

TABLE III.

The Melting Point of the Amino Hydrate Form.

No. of sample.	Slight browning.	Sublime or mist.	First droplets.	Completely melted.	Froth.
129			214	216	216.5
114			207	212	214

TABLE IV.

The Melting Point of the Amino Carboxyl Salt Form.

No. of sample.	Slight browning.	Sublime or mist.	First droplets.	Completely melted.	Froth.
86	215		219	220	221
88			216	218	221
89			216	218	219
95			219.5	221	222
100			222	223.5	223.5
105			222	223	223.5
112			216	220	221
113			217	221	222
118			221	222	222.5
120			221	224	224
121			222	224	225
127			218	222	223
130				225.5	226

TABLE V.

The Melting Point of Acid Salts of the Amino Group.

	No. of sample.	Slight browning.	Sublime or mist.	First droplets.	Completely melted.	Froth.
Amino sulfate.....	83		182	207	208	208.5
" "	93	162		202	204	204
" "	103	180		206	207	208
" oxalate.....	104	180		206	207	208
" formate.....	101			202	205	208
" "	76	150		190	205	206.5
" "	84		180	202	204	204
" "	82	160			204	205
" acetate.....	102			202	205	208
" carbonate.....	87	160		204	206	206

TABLE VI.

The Melting Point of the Acetyl.

No. of sample.	Slight browning.	Sublime or mist.	First droplets.	Completely melted.	Froth.
34			220	228	230
30			225	235	236
29			215	223	232

TABLE VII.

The Melting Point of the Sulfate of the Acetyl.

No. of sample.	Slight browning.	Sublime or mist.	First droplets.	Completely melted.	Froth.
27			111	150	200
25				122	190
24			100	106	150

TABLE VIII.

The Melting Point of the Acetyl in Open-Ring Form.

No. of sample.	Slight browning.	Sublime or mist.	First droplets.	Completely melted.	Froth.
8			150	160	174
35				155	155
11			146	152	216

TABLE IX.

The Melting Point of the Ureide.

No. of sample.	Slight browning.	Sublime or mist.	First droplets.	Completely melted.	Froth.
21				225	225

SUMMARY.

The most important physical and chemical properties of thyroxin may be summarized as follows:

1. Thyroxin is a colorless, odorless, crystalline substance, insoluble in aqueous solutions of all acids including carbonic. It is soluble in sodium, ammonium, and potassium hydroxides, and is very slightly soluble in sodium and potassium carbonate. Besides forming salts with metals, thyroxin also forms salts with acids.

2. The iodine content of thyroxin and the iodine content of the sulfate salt were found to be 65 and 60 per cent respectively. This established the molecular weight of 585. Ultimate analysis and a study of the derivatives of thyroxin show the structural formula to be 4, 5, 6 tri-hydro-4, 5, 6 tri-iodo,-2 oxy,-beta indolepropionic acid.

3. In the presence of alkali metal hydroxides, thyroxin forms di-basic salts through the carboxyl and hydroxy groups. In the presence of carbonates, thyroxin forms mono-basic salts with the carboxyl group alone. The imino group forms salts with mineral and formic acids but not with acetic. The salts of mineral acids are soluble in alcohol, but no acid salt of thyroxin is appreciably soluble in water. Thyroxin forms derivatives through the imino nitrogen, such as the acetyl and ureide, and through its carboxyl and hydroxy groups, such as the dimethyl derivative.

4. Thyroxin exists in four distinct forms: (1) The keto form with the imino carbonyl groups, melting point 250°; (2) the enol form in which the hydrogen migrates from the imino to the carbonyl forming the hydroxy group, melting point 204°; (3) an open-ring form in which the elements of water enter the molecule between the imino and carbonyl groups forming an open-ring structure with amino and carboxyl groups, which exist in salt formation, called the amino carboxyl salt form, melting point 225°; and (4) a tautomeric form of this in which the elements of water

add to the nitrogen making the amino hydrate form, melting point 216°. If an acid is added to an enol form of thyroxin, the ring opens and the acid forms an amino-acid salt. The reason why weak organic acids including carbonic can add to the nitrogen of thyroxin-forming amino salts is because the ring is unstable in neutral aqueous solutions and the nitrogen tends to exist in the pentad state adding either the elements of water and forming an amino hydrate, or adding a carboxyl and forming an amino salt. These reactions could occur only with a strongly basic group. The amino group of analine and the imino group of indole or isatin are too feebly basic to react the same as thyroxin with weak organic acids.

5. Thyroxin is not easily oxidized or reduced, but will yield to both oxidation and reduction if sufficiently strong agents are used.

6. In alkaline solutions the iodine is broken off from the thyroxin molecule not as free iodine but as hypoiodous acid. This reaction is accelerated by sunlight. Sunlight also produces pink color compounds from the colorless thyroxin molecule.

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CARBONIC ACID AND CARBONATES IN COW'S MILK.

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(Received for publication, July 26, 1919.)

The amount of carbonic acid present in cow's milk has been the subject of several investigations.[1-5] The results reported by these investigators vary from 1.84 to 7.65 per cent of CO_2 by volume. It is not necessary to review the methods which have been employed to obtain the CO_2 from milk for measurement farther than to say that they have varied from extraction by means of a vacuum pump to expulsion by heat and have been more or less open to inaccuracy; nor is it important to consider previous methods used in measuring the amount of CO_2.

The results of our work are presented under the following divisions: (I) determination of CO_2 in milk; (II) relation of pasteurized milk to CO_2; (III) the form in which CO_2 exists in milk; (IV) the tension of CO_2 in milk.

I. Determination of CO_2 in Milk.

In measuring the amount of CO_2 in milk, we have used Van Slyke's method[6,7] in the determination of CO_2 in blood plasma, with certain modifications required to adapt the method to conditions present in milk.

[1] Hoppe, F., *Virchows Arch. path. Anat. u. Physiol.*, 1859, xvii, 417.

[2] Setschenow, *Z. ration. Med.*, 1861, x, 285.

[3] Pflüger, E., *Arch. ges. Physiol.*, 1869, ii, 166.

[4] Thörner, W., *Chem. Z.*, 1894, xviii, 1845.

[5] Marshall, C. E., *Michigan Agric. Exp. Station, Special Bull. No. 16.*, 1902.

[6] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

[7] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 291.

Our method of procedure will be described under the five heads following: (1) drawing milk from cow's udder; (2) sampling the milk drawn; (3) determination of CO_2; (4) results obtained with different milks; (5) determination of CO_2 in milk by exhaustion.

1. Method of Drawing Milk.—In order to prevent loss of CO_2, it is of primary importance to have under complete control the method of drawing from the cow's udder the sample of milk in which CO_2 is to be measured. We have found the following method satisfactory: A silver milking-tube is inserted into the teat and the milk flows readily from the udder through this tube. To prevent the milk from coming into contact with the air, it is collected in a 100 cc. cylinder provided with a close fitting two-hole rubber stopper. Through one hole passes a glass tube extending to the bottom of the cylinder, and this tube is connected at its upper end by rubber tubing with the milking-tube. Passing through the second hole of the rubber stopper is a short glass tube which serves as an outlet for the air in the cylinder while it is being filled with milk. With this arrangement, the milk flows slowly and quietly into the cylinder without exposure to the air. The cylinder fills from the bottom upward, displacing the air until the milk fills the cylinder completely or even to slight overflowing. If desired, the surface of the milk can be protected from the air by a layer of thin paraffin oil but we have not found this necessary.

2. Taking Sample for CO_2 Determination.—It is essential that the samples for the determination of CO_2 be taken from the cylinder as soon as practicable after the milk is drawn from the udder, because, on standing, the rising of the fat-globules to the surface changes the composition of the different layers of milk, and also there may be diffusion of the CO_2 from the upper surface if the top of the cylinder is not entirely filled with milk. The best method of taking the sample from the cylinder is the following: One end of a 2 cc. pipette is connected to the tube leading to the bottom of the cylinder. Then one blows into the short tube, forcing the portion of milk last drawn from the udder to flow into the pipette until the milk overflows from the pipette, displacing all the air. The sample then passes from the lower part of the cylinder into the pipette without exposure to the air, without loss of CO_2, without being subject to appreciable change

of pressure, and without disturbance of the body of the milk in the cylinder. It has been found that the portion of milk first drawn from the udder and occupying the upper portion of the cylinder does not fairly represent the CO_2 content of the entire milking.

3. Determination of CO_2.—The 2 cc. sample of milk is run into the Van Slyke CO_2 apparatus. Only slight modifications are required in the method as described by Van Slyke. We find that 5 per cent sulfuric acid and other mineral acids coagulate the milk and the coagulum interferes with the operation by clogging the apparatus, making it difficult to remove the CO_2 completely from the mixture. Concentration of such acids greater than 5 per cent was also found unsatisfactory. As the result of numerous experiments with different acids and concentrations, we find that a 20 per cent solution of lactic acid obviates all difficulties and meets all requirements, dissolving the casein quickly and completely and giving the same corrections as apply to the use of 5 per cent sulfuric acid. In all our work here reported, the amount of CO_2 was determined by absorption with a 5 per cent solution of NaOH instead of making a correction for inert gases. The results are the same by either process.

4. Results Obtained with Different Milks.—In Table I we give the results furnished by the examination of twenty-five samples of cow's milk, drawn from the individual quarters of the udder. In addition to the amount of CO_2 in the milk, we give in each case the pH value and also the titration value expressed as cc. of alkali required to neutralize 100 cc. of milk to phenolphthalein. The results are arranged in the order of the pH values.

A study of the data embodied in Table I leads to the following statements:

1. With the increase of the value of pH, that is, with decrease of hydrogen ion concentration, there is a general tendency for the CO_2 content of the milks to increase and for the degree of acidity, as measured by titration, to decrease. This is not so marked between the pH values of 6.50 and 6.65 as it is above pH 6.65. Below pH 6.65, the CO_2 content varies between 7 and 10 per cent, while above pH 6.65 it increases somewhat uniformly from 12 at pH 6.70 to 86 at pH 7.16.

2. The acidity, as measured by titration, varies below pH 6.65 more or less irregularly between 16 and 20 cc. of 0.1 N alkali per 100 cc. of milk, while above pH 6.65, the values decrease quite uniformly from 16 down to 4 cc.

3. In comparison with the values obtained by other workers, our lowest CO_2 values are about equal to, or higher than, the highest values previously reported. Thus, the highest figure heretofore published is 7.65 per cent of CO_2 by volume, while most of our values range from 8 up to 56 per cent in milks which

TABLE I.

Amount of CO_2 in Cow's Milk.

CO_2 (corrected) by volume.	pH value.	0.1 N alkali required to neutralize 100 cc. of milk.	CO_2 (corrected) by volume.	pH value.	0.1 N alkali required to neutralize 100 cc. of milk.
per cent		*cc.*	*per cent*		*cc.*
8	6.50	19.1	9	6.62	17.2
7	6.52	20.0	10	6.63	18.0
8	6.53	18.0	10	6.65	16.6
10	6.54	16.0	12	6.70	15.4
10	6.55	17.2	12	6.80	16.0
8	6.55	18.4	18	6.82	13.0
11	6.57	17.2	14	6.86	14.0
10	6.58	17.8	22	6.90	12.0
10	6.58	16.9	33	6.92	12.0
10	6.58	18.4	24	7.00	10.0
9	6.60	18.2	56	7.05	6.0
10	6.61	16.8	86	7.16	4.0
10	6.62	17.8			

appeared by ordinary inspection to be normal. In the milks examined by us which were known to be normal, the value most frequently found is about 10 per cent. However, our work has not been sufficiently extensive as yet to enable us to indicate positively a general average figure or an average range for normal milk.

5. Determination of CO_2 in Milk by Exhaustion.—It was desirable to ascertain whether it is possible to remove the CO_2 completely from milk for determination simply by vacuum exhaustion. We have found that this can be done by observing certain precautions. The milk must be spread out in a thin layer during the

exhaustion. We placed 10 cc. of milk of the usual reaction (pH 6.5 to 6.65) in a 200 cc. separatory funnel and exhausted this for 2 minutes, turning the funnel end over end slowly in order to spread the milk in a thin layer over the interior surface of the funnel as completely as possible. Air was then admitted and the exhaustion repeated, after which the determination of CO_2 in the sample was made.[8]

Another portion (100 cc.) of the same milk was then placed in a 200 cc. separatory funnel and inverted. This was exhausted for 1 hour without any agitation of the milk, after which the amount of CO_2 in the sample was determined. We give the results of the two experiments in Table II.

TABLE II.

Results of Removal of CO_2 from Milk by Vacuum Exhaustion.

Original milk.		After exhaustion without agitation.		After exhaustion with agitation in a thin layer.	
pH value.	CO_2 by volume.	pH value.	CO_2 by volume.	pH value.	CO_2 by volume.
	per cent		*per cent*		*per cent*
6.54	10	6.57	4	6.60	0.0
6.86	14	6.92	7	6.98	0.0
6.92	22	7.00	9	7.06	0.0

These results make prominent certain points, as follows:

1. The CO_2 of milk can be completely removed by vacuum exhaustion, as shown by the results given in the last column of Table II, provided the milk is agitated and kept in a thin layer, the amount of milk used being small enough to permit control of these conditions. If the milk subjected to exhaustion is not agitated and exposed in a thin layer, the CO_2 is not completely removed, as shown by the results given in the fourth column of Table II. In previous work done by others, in which vacuum exhaustion was relied upon to remove the CO_2 from milk, the removal was incomplete, owing to failure to observe the conditions required for complete exhaustion, as in the case of the results reported by Setschenow[2] and by Marshall.[5]

[8] Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 369.

2. The removal of CO_2 from milk results in an increase in the value of pH, that is, a decrease in the hydrogen ion concentration or, stated in another way, the milk becomes less acid, though to an amount that cannot be made appreciable by titration in normal milks. In the first sample in Table II, the original milk, containing 10 per cent by volume of CO_2 has a pH value of 6.54, which increases to 6.57 when the CO_2 is reduced to 4 per cent, and which increases farther to pH 6.60 when the CO_2 is completely removed.

II. Relation of Pasteurized Milk to CO_2.

The observation stated in the preceding paragraph led us to make a study of some results which we had obtained in another investigation relating to the effect of pasteurization upon the reaction of milk. We had noticed that pasteurization, if properly performed, is without observable effect in changing the hydrogen ion concentration of milk. Our experiment was repeated with the modification that the CO_2 was completely removed from the milk by exhaustion before heating. The two experiments gave results as recorded in Table III.

TABLE III.

Effect of Removal of CO_2 on Reaction of Pasteurized Milk.

Before removal of CO_2.				After removal of CO_2.			
Before heating.		After heating at 63°C. for 15 min.		Before heating.		After heating at 63°C. for 15 min.	
pH value.	CO_2 by volume.	pH value.	CO_2 by volume.	pH value.	CO_2 by volume.	pH value.	CO_2 by volume.
	per cent		*per cent*		*per cent*		*per cent*
6.54	10	6.54	2	6.60	0	6.56	0

In studying the results of these two experiments, we notice:

1. In the milk in which CO_2 is not removed before heating, the pH value remains the same before and after heating, even though the per cent of CO_2 is decreased by the heating from 10 to 2 per cent.

2. In the milk in which CO_2 is completely removed by exhaustion before heating, the pH value decreases from 6.60 to 6.56, that is, the hydrogen ion concentration increases appreciably.

3. We have not yet carried our work far enough to furnish an explanation of the fact noted, but the inference appears justified that some chemical change occurs in the milk during pasteurization which results in an increase of hydrogen ion concentration, when CO_2 is absent, but that in the presence of CO_2 any change in the hydrogen ion concentration is, in some way not yet known, masked or offset by the loss of CO_2 which escapes from the milk during heating.

4. The decrease of CO_2 in pasteurized milk suggests that the CO_2 content of milk might be made the basis of a method for distinguishing pasteurized from normal milk. We are doing additional work in order to determine the limits of effectiveness of such a method.

III. Form in which CO_2 Exists in Milk.

It has been generally assumed that CO_2 exists in milk as uncombined carbonic acid. From the fact that the reaction of milk is less acid than that given by a corresponding solution of CO_2 in water, it appears probable that the CO_2 in milk is present in part as carbonic acid and in part as bicarbonate. To determine the proportion of CO_2 existing in milk as carbonic acid and as bicarbonate, two methods are available; (1) by calculation based on the application of the law of mass action, and (2) by direct determination. The results obtained by either of these methods can be regarded as only approximate, owing to the high dilution of CO_2 in milk.

1. Calculation Based on Application of the Law of Mass Action.— In a solution containing H_2CO_3 and $RHCO_3$, there exist in accordance with the law of mass action definite quantitative relations between the hydrogen ion concentration of the solution and the relative amounts of H_2CO_3. These relations are expressed by the following equations:

$$C_H = K \frac{H_2CO_3}{CO_3} = K \frac{H_2CO_3}{\alpha RHCO_3},$$

in which α is the degree of dissociation of $RHCO_3$ into R^+ and HCO_3, and K is the ionization constant of H_2CO_3. From the foregoing, we have $\frac{H_2CO_3}{RHCO_3} = \frac{\alpha C_H}{K}$. Therefore, to determine the ratio

between bicarbonate and carbonic acid, we need only to know the values of K, C_H, and α for $RHCO_3$ (as $NaHCO_3$). According to Michaelis and Rona,[9] K equals 4.4×10^{-7}; the C_H value of average milk is about 0.25×10^{-6}; the value of α is difficult to determine with more than an approximate degree of accuracy under the conditions present in milk, but by a method similar to that of Michaelis and Rona, we obtain a value which makes the ionization of the bicarbonate in milk about 80 per cent. Applying these values in the equation, $\frac{H_2CO_3}{RHCO_3} = \frac{\alpha C_H}{K}$ we have $\frac{0.80 \times 0.25 \times 10^{-6}}{4.4 \times 10^{-7}} = \frac{10}{22}$. This result means that the CO_2 exists in milk in approximately the relation of one part of H_2CO_3 for two parts of bicarbonate, or that one-third of the CO_2 exists as H_2CO_3 and two-thirds as bicarbonate.

2. Proportion of Bicarbonate Acid and Bicarbonate Determined by Experiment.—The second method of ascertaining the proportion of CO_2 in milk present as carbonic acid and bicarbonate is based on the isohydric principle. A solution of carbonate containing a molecular concentration equal to that of milk would, if adjusted to the same hydrogen ion concentration, have approximately the same relative proportions of carbonic acid and bicarbonate. Milk is approximately a 0.01 N solution of H_2CO_3. In carrying out the details of our experiment, we dilute 10 cc. of a 0.1 N solution of Na_2CO_3 to 100 cc. with water free from CO_2. Then we add a solution of 0.1 N HCl until the reaction is the same as that commonly found in milk (C_H, 0.25×10^{-6}). This requires 6.6 cc. of the acid. Of the 6.6 cc. of the 0.1 N HCl thus required 5 cc. are used to change Na_2CO_3 into $NaHCO_3$, leaving 1.6 cc. of 0.1 N HCl to act upon the $NaHCO_3$ and form H_2CO_3. In changing the 100 cc. of 0.01 N Na_2CO_3 solution into $NaHCO_3$, the resulting 100 cc. of 0.01 N $NaHCO_3$ has only one-half the neutralizing power of the Na_2CO_3 solution. Therefore, 1.6 cc. of 0.1 N HCl neutralizes 3.2 cc. of the 100 cc. of 0.01 N $NaHCO_3$ solution, forming 32 cc. of 0.01 N H_2CO_3 and leaving 68 cc. of 0.01 N $NaHCO_3$. These results furnish the ratio, 32 H_2CO_3: 68 $NaHCO_3$, or, approximately, the ratio of 1 : 2 ; that is, one-third

[9] Michaelis, L., and Rona, P., *Biochem. Z.*, 1914, lxvii, 182.

part of the CO_2 exists in the solution as H_2CO_3 and two-thirds as $NaHCO_3$, a result which is in close agreement with that obtained by application of the law of mass action.

In this connection it is interesting to note that Marshall[9] states that CO_2 is not completely removed from milk by his method of vacuum exhaustion, owing, as he seems to think, to a slow generation of CO_2 in the milk. This is readily explained by the presence of bicarbonate in milk, which gradually gives up its CO_2 under reduced pressure as a result of the reaction of bicarbonate with some of the salts contained in milk.

IV. The CO_2 Tension in Milk.

The CO_2 tension in milk is about the same as in most fluids of the animal body. Using McClendon's chart[10] we find by extrapolation of values that the CO_2 tension at 20°C. of a 0.01 N solution, pH 6.6, is approximately equal to 50 to 55 mm. of mercury. It is a matter of interest to notice that the CO_2 tension of blood under the conditions is given by him as 47 mm. Comparing this with the value for milk, one would expect a lower value in blood, because the latter is exposed to air in the lungs and, therefore, subject to loss of CO_2 by removal.

SUMMARY.

1. Milk is drawn from the cow's udder into a 100 cc. cylinder so as to fill the cylinder from the bottom upward, thus avoiding mixture with air or loss of CO_2. For the determination of CO_2, 2 cc. of milk are forced from the cylinder into the Van Slyke CO_2 apparatus without loss of CO_2. A 20 per cent solution of lactic acid is used to free the CO_2 in carbonates.

2. In the case of twenty-five samples of milk drawn from separate quarters of the udder, the CO_2 varies from 7 per cent by volume to 86 per cent; the pH value varies from 6.50 to 7.16, in a general way increasing with the CO_2 content; the degree of acidity, as measured by titration, tends to decrease with increase of CO_2 content. In comparison with the results of other workers, the results obtained by us are higher. The CO_2 content of normal milk appears to be about 10 per cent by volume.

[10] McClendon, J. F., *J. Biol. Chem.*, 1917, xxx, 274.

3. It is possible to remove CO_2 from milk completely by vacuum exhaustion provided the milk is spread in a thin layer and kept in motion.

4. When milk is pasteurized, the CO_2 content is decreased, but the pH value remains unchanged. However, if the CO_2 is completely removed before pasteurization, then the pH value appears to decrease slightly after pasteurization.

5. CO_2 exists in milk as H_2CO_3 and as bicarbonate, probably $NaHCO_3$, the ratio being about one part of H_2CO_3 and two parts of $NaHCO_3$.

6. The CO_2 tension in milk is calculated to be about equal to 50 to 55 mm. of mercury at 20°C. in case of a 0.01 N solution with a pH value of 6.60.

CONDITIONS CAUSING VARIATION IN THE REACTION OF FRESHLY-DRAWN MILK.

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(Received for publication, July 26, 1919.)

The most sensitive method of measuring the reaction of milk is by measurement of the hydrogen ion concentration. This method has been employed by several investigators[1-7] in connection with the study of milk and it has been utilized in the work here presented.

The results of previous investigations show cow's milk, when freshly drawn, to have a reaction, expressed in terms of pH values, varying from 6.39 to 6.81, the range in most cases being between 6.50 and 6.65.

It appeared to us desirable to ascertain the extent of variation in large numbers of milks obtained directly from cows and to learn further, if possible, the causes of such differences. The results of our work will be discussed under the following headings: (1) Extent of variation of reaction in cow's milk, (2) variation in different quarters of the udder, (3) relation of reaction of milk to composition, (4) effect of abnormal conditions in the udder.

Extent of Variation of Reaction in Cow's Milk.

In undertaking to establish in our own experience the extent to which the reaction of fresh milk varies, we measured the hydrogen ion concentration in over 300 samples obtained from two herds of

[1] van Dam, W., *Rev. gén. Lait*, 1908, vii, 121.
[2] Allemann, O., *Biochem. Z.*, 1912, lxv, 346.
[3] Taylor, H. B., *J. Proc. Roy. Soc. N. S. Wales*, 1913, lxvii, pt. 2, 174.
[4] Davidsohn, H., *Z. Kinderheilk.*, 1913, ix, 14.
[5] Clark, W. M., *J. Med. Research*, 1914–15, xxxi, 431.
[6] Milroy, T. H., *Pharmacol. J.*, 1914, xciii, 350.
[7] Foa, C., *Compt. rend. Soc. biol.*, 1904, lix, 51.

cows; one was a herd of Jerseys and the other of Holstein-Friesians. The samples used in our work were drawn separately from the individual quarters of the udder and the reaction was determined in each. In obtaining the samples, the first few streams of milk were thrown away and then enough more was drawn for use in our experiments. The results do not, therefore, represent the complete milking of the entire udder but only the foremilk of each quarter of the udder. The use of milk from individual quarters of the udder gives a greater variation in results than would be found if we used only samples representing the mixed milk of a complete milking of the entire udder. The use of the foremilk is very convenient for our purpose and is justifiable, since in our experience the reaction rarely changes appreciably in normal milks in portions successively drawn during milking, provided there is no excessive disturbance of the udder.

In Table I we give summarized results of our work with over 300 samples of milk, the reaction being stated in terms of pH values.

TABLE I.

		pH 6.50 to 6.60.	pH 6.60 to 6.68.	pH 6.68 to 6.76.	pH 6.76 to 6.84.	pH 6.84 to 6.92.	pH 6.92 to 7.00.	pH 7.00 to 7.20.	Total number of samples.
Herd 1..	Number of samples.	78	35	23	7	6	2	2	153
	Per cent of total.	51	22.9	15	4.6	3.9	1.3	1.3	—
Herd 2..	Number of samples.	61	32	27	15	9	6	5	155
	Per cent of total.	39.4	20.6	17.4	9.7	5.8	3.9	3.2	—

The samples of milk from the two herds of cows show quite as wide a range of hydrogen ion concentration as we have found in our entire experience up to the present time, working with a great variety of milks, though it is probable that somewhat wider variations may occur. Our results indicate that the reaction of fresh normal milk, expressed in terms of pH values, lies between 6.50 and 6.75 or 6.80. In Herd 1, 136 samples, or nearly 90 per cent of all the samples, and in Herd 2, 120 samples, or over 77 per cent, are below pH 6.76, that is, an average of 83 per cent of all

the samples examined for these two herds. These figures are in agreement with results previously published, except that we find a small proportion of milks which are less acid, the pH values reaching as high as 7.2. We usually find that milks of such abnormally low acidity are sufficiently normal in appearance to pass the ordinary methods of market inspection when mixed in the commercial supply with other milks that are normal. The wide range of values obtained by us is doubtless due to the fact that the samples of milk used in our work represent individual quarters of the udder and not the complete mixed milk drawn from the entire udder at one milking. The mixed milk of a herd shows still smaller variation than that from single cows.

Variation of the Reaction of Milk in Different Quarters of the Udder.

It is a matter of interest to show at this point to what extent milk drawn from different quarters of a cow's udder may vary in reaction. In Table II we give results obtained with twenty cows. The hydrogen ion concentration in these samples was determined approximately by means of brom-cresol purple used as an indicator, as described on page 364. The results are given in two forms. The range of pH values from 6.50 to values above 7 is divided into seven groups, indicated by number, and after each such group number there is given in parenthesis the corresponding range of specific values.

A study of the tabulated data suggests the following points of interest.

1. The reaction found to be the more common is that showing the higher acidity. Out of the 80 samples of milk drawn from the quarters of the udders of the twenty cows used in the work, 39, or nearly 50 per cent, are in Group 1, showing the highest acid reaction (pH 6.50 to 6.60); 21, or over 26 per cent, are in Group 2 (pH 6.60 to 6.68); 14, or over 17 per cent, are in Group 3 (pH 6.68 to 6.76); these three groups contain 92.5 per cent of the total. Group 4 (pH 6.76 to 6.84) contains three samples, while the least acid groups, 5, 6, and 7, contain only one sample each.

2. These results indicate that the hydrogen ion concentration of normal mixed milk, when fresh, is that representing the most

acid reaction found by us (pH 6.50 to 6.60), the variations being in the direction of decreased acidity.

3. Comparing the different quarters of the udder in individual cows, we find that there are only four (Nos. 16, 19, 20, 14) in which the reaction is the same in all quarters; and in these cases the reaction is that of the most acid groups, 1 and 2. In another examination of the same twenty cows, ten individuals were found to give a uniform reaction in the milk from all quarters, eight showing pH 6.50 to 6.60 and two, pH 6.60 to 6.68. In Table II the milk of only five animals (Nos. 12, 11, 18, 7, 3) departs markedly from the normal reaction, and in these cases the ab-

TABLE II.

Results Showing Variation in Reaction of Milk Drawn from Different Quarters of Udder.

Cow No.	Quarter of udder.							
	Right front.		Left front.		Right hind.		Left hind.	
	Group.	pH	Group.	pH	Group.	pH	Group.	pH
16	1	(6.50–6.60)	1	(6.50–6.60)	1	(6.50–6.60)	1	(6.50–6.60)
19	1	" "	1	" "	1	" "	1	" "
20	1	" "	1	" "	1	" "	1	" "
14	2	(6.60–6.68)	2	(6.60–6.68)	2	(6.60–6.68)	2	(6.60–6.68)
2	2	" "	1	(6.50–6.60)	1	(6.50–6.60)	1	(6.50–6.60)
13	1	(6.50–6.60)	2	(6.60–6.68)	1	" "	1	" "
15	1	" "	3	(6.68–6.76)	1	" "	1	" "
10	3	(6.68–6.76)	2	(6.60–6.68)	2	(6.60–6.68)	2	(6.60–6.68)
12	5	(6.84–6.92)	3	(6.68–6.76)	3	(6.68–6.76)	3	(6.68–6.76)
1	2	(6.60–6.68)	2	(6.60–6.68)	1	(6.50–6.60)	1	(6.50–6.60)
5	2	" "	1	(6.50–6.60)	1	" "	2	(6.60–6.68)
9	3	(6.68–6.76)	3	(6.68–6.76)	1	" "	1	(6.50–6.60)
4	1	(6.50–6.60)	1	(6.50–6.60)	2	(6.60–6.68)	3	(6.68–6.76)
8	2	(6.60–6.68)	2	(6.60–6.68)	1	(6.50–6.60)	3	(6.68–6.76)
6	2	" "	3	(6.68–6.76)	1	" "	2	(6.60–6.68)
17	3	(6.68–6.76)	2	(6.60–6.68)	3	(6.68–6.76)	1	(6.50–6.60)
11	4	(6.76–6.84)	1	(6.50–6.60)	3	(6.68–6.76)	1	" "
18	1	(6.50–6.60)	1	" "	4	(6.76–6.84)	3	(6.68–6.76)
7	2	(6.60–6.68)	1	" "	1	(6.50–6.60)	6	(6.92–7.00)
3	1	(6.50–6.60)	2	(6.60–6.68)	7	(7.00–7.20)	4	(6.76–6.84)

normal condition appears in only six of the twenty individual quarters.

4. While it is not the purpose of this article to discuss in detail the causes of these observed variations in reaction in the milk from the different quarters of the udder, we may state in passing that such variations must be due to some physiological condition of the animal, either a specific bacterial infection of the udder or a more general constitutional condition, such as variation in base and acid relations in the blood stream.

Relation of the Reaction of Milk to Composition.

Attention has been called to the fact that the reaction most commonly prevalent in freshly-drawn milk is the one that is most acid, and that the variation is all in the direction of decreased acidity; and, further, that the number of samples in which the acidity decreases is found to fall off rapidly with the greater decrease of acidity.

It was desired to ascertain, if possible, some of the conditions under which decrease of acidity occurs. Our attention was first turned to a study of possible relations that might exist between changes in reaction and changes in composition of milk. We made analyses of several samples of freshly-drawn milk in the case of cows which had previously been found to give milk varying noticeably in reaction from normal. Each sample of milk was drawn from one quarter of the udder, the entire contents being drawn, except in certain cases to which attention is called. All the samples were from cows whose milk was going into the local market supply. There was nothing abnormal that was observable in the appearance of the milk or of the cows, except that the milk with the least acid reaction had the characteristic bluish appearance of what we commonly call "thin" or "poor" milk.

The results are given in Table III, the analyses being arranged in the order of pH values, beginning with the most acid.

An examination of the data in Table III suggests that there are certain points of correspondence between the reaction of milk and the composition.

We observe that, with a decrease of acidity, there is a marked tendency toward a decrease in the specific gravity and in the per-

centage of total solids, fat, solids-not-fat, casein, and sugar; but, on the other hand, an increase in albumin and proteins other than casein, and in the ash and also in the chlorine. There is, further, as we have shown in another article (p. 338) an increase in CO_2 content with decrease of acidity.

TABLE III.

Results Showing Reaction of Milk in Relation to Composition.

Cow No.	pH value.	Total solids.	Fat.	Total proteins.	Casein.	Proteins other than casein.	Sugar.	Ash.	Chlorine.	Specific gravity.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
1	6.53	12.81	4.50	3.21	2.55	0.66	4.60	0.70	0.09	1.029
2	6.56	12.07	3.60	3.32	2.56	0.76	4.60	0.65	0.12	1.031
3	6.58	14.57	5.40	3.60	2.75	0.85	5.10	0.72	0.10	1.032
4*	6.60	13.30	3.80	3.31	2.62	0.69	5.20	0.69	0.11	1.030
5	6.70	12.10	3.60	3.21	2.50	0.71	4.40	0.86	0.13	1.030
6†	6.80	13.29	4.30	4.49	2.84	1.65	3.70	0.71	0.14	1.028
7	6.85	13.74	5.40	3.00	2.20	0.80	4.50	0.81	0.14	1.030
8	6.91	9.41	1.20	3.02	2.05	0.97	4.16	0.79	0.14	1.030
9	6.95	10.58	2.40	3.14	2.05	1.09	3.80	0.83	0.16	1.030
10	6.96	10.48	3.40	2.45	1.69	0.76	3.80	0.83	0.14	1.026
11	6.98	10.41	2.80	2.80	1.79	1.01	4.00	0.81	0.15	1.027
12‡	7.00	12.42	5.65	2.73	1.63	1.10	3.20	0.84	0.18	1.022
13	7.04	10.10	2.20	3.23	2.26	0.97	3.70	0.84	0.16	1.030
14*	7.06	8.85	2.80	2.64	1.50	1.14	2.60	0.91	0.22	1.021
15	7.15	9.13	2.10	3.14	1.71	1.43	3.00	0.89	0.21	1.026

* The sample was the last portion of milk drawn from the udder (strippings).

† The sample was from a cow in the last stage of lactation, being nearly "dry."

‡ The sample was from a cow just beginning the period of lactation, or "fresh in milk."

These findings raise the question as to whether there is any reason for the correspondence existing between the observed changes in reaction and composition. These changes are such as would be expected, if we were to add blood-serum or lymph to normal milk and they are also in agreement with the results re-

ported by others[8–16] who have worked with milk from diseased udders, though our samples were from udders which were apparently in normal condition.

This phase of the question brings us to a consideration of abnormal conditions of the udder in relation to the reaction of milk.

The Reaction of Milk in Relation to the Presence of Leucocytes and Bacteria in the Udder.

All the samples of milks used by us were from udders which were apparently in a condition of normal health under casual observation; but a special examination of those samples (Table III) showing a reaction indicated by pH values above 6.70 was made for leucocytes and streptococci. For the work done in making these examinations, we are under obligation to Miss Mildred C. Davis, City Bacteriologist of Geneva. Use was made of Breed's[17] method of direct-counting in the milk. The results of the work are given in Table IV.

The results in Table IV indicate in a general way that decreased acidity in fresh milk is related to infection of the udder. Decrease of acidity is shown to be associated with increase of leucocytes, provided acid-producing streptococci are not present in sufficient numbers to neutralize such effect. Thus, in No. 7, we have a milk not far from normal in reaction, even though it contains a large number of leucocytes (20 million per cc.), a number which in Samples 14 and 15 gives a marked relative decrease in acid reaction; but this condition in No. 7 appears to be accounted for by the relatively large number of acid-producing streptococci (1 million per cc.), the acid produced offsetting the decrease of acidity caused by leucocytes.

[8] Storch, V., *Jahresb. Thierchem.*, 1884, xiv, 170; 1889, xix, 157.
[9] Hoyberg, H. M., *Z. Fleisch.-u. Milchhyg.*, 1911, xxi, 1.
[10] Fetzer, L. W., *Eighth Internat. Cong. Applied Chem.*, 1912, xix, 111.
[11] Chrétien, M., *Hyg. viande et lait*, 1912, vi, 382.
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[13] Zaribnicky, F., *Arch. wissensch. u. prakt. tierheilk.*, 1913–14, xl, 355.
[14] Henderson, J. B., and Meston, L. A., *Chem. News*, 1914, cx, 275, 283; 1915, cxi, 51.
[15] Bahr, L., *Z. Fleisch.-u. Milchhyg.*, 1913-14, xxiv, 251, 288, 370, 398, 472.
[16] Foa, C., *Compt. rend. Soc. biol.*, 1905, lix, 51.
[17] Breed, R. S., *New York Agric. Exp. Station, Techn. Bull. 49*, 1916.

TABLE IV.

Relation of Leucocytes and Streptococci to the Reaction of Milk.

Cow No.	pH value.	No. of leucocytes per cc.	No. of streptococci per cc.
		millions	*millions*
6	6.80	1	0.1
7	6.85	20	1.0
8	6.91	3	Small number.
9	6.95	5	" "
10	6.96	10	" "
11	6.98	8.4	" "
12	7.00	4	0.2
13	7.04	3	Small number.
14	7.06	20	0.5
15	7.15	21	0.3

A further study was made of five samples of milk in which the approximate pH value was determined by means of brom-cresol purple used as an indicator, the microscopic examination for leucocytes and streptococci being made by Miss Davis. The results are given in Table V.

TABLE V.

Results of Examination of Milk for Leucocytes and Streptococci.

Sample No.	Approximate pH value.	Cells.	Acid production.
1	6.68–6.76	Many leucocytes. Streptococci, innumerable and in clumps at bottom of tube.	Marked acid production by clumps at bottom of tube within 1 hour after milking.
2	6.72–6.80	5 million leucocytes per cc. Streptococci, innumerable, separate, and in clumps.	Marked acid production by clumps at bottom of tube within 30 minutes after milking.
3	6.76–6.84	Many leucocytes. Streptococci, innumerable, separate, and in clumps at bottom of tube.	Marked acid production by clumps at bottom of tube within 30 minutes after milking.
4	Over 7.00	Leucocytes, innumerable; few streptococci.	No acid production apparent.
5	" 7.00	Leucocytes, innumerable; few streptococci.	No acid production apparent.

Summarizing the results embodied in Table V, we notice:

1. In Samples 4 and 5, containing innumerable leucocytes and showing evidence of the presence of garget, there were few streptococci and the acidity was lowest. On standing a few hours, these samples furnished no evidence of increase of acidity.

2. In Samples 1, 2, and 3, which were the more acid ones, there were immense numbers of streptococci, especially in clumps; and while there were also many leucocytes, there was an increase of acidity within a very short time after the milk was drawn from the udder.

3. It appears highly probable that the greater acidity in the milks containing enormous numbers of streptococci is due to the formation of acid by these organisms, especially in view of the fact that milk from diseased udders, containing large numbers of leucocytes with few streptococci, shows the lowest degree of acidity found in fresh milk. In other words, we find milks that contain large numbers of leucocytes and are abnormally low in acidity when streptococci are present in only small numbers or entirely absent usually show appreciably higher acidity when streptococci are present in large numbers.

A Suggested Explanation of the Decreased Acidity in Abnormal Milks.

We have already stated that the correspondence existing between the observed changes in reaction and composition of milk may be accounted for on the supposition that the change in the direction of decreased acidity is due to the presence of blood-serum or lymph. Decrease of acidity in fresh milk is observed in case of diseased udders and may, therefore, be due to the direct filtration of blood-serum or lymph into the lumen of the alveoli without transformation by the gland cells or through lesions caused by bacterial activity. This view harmonizes with several facts. (1) It is in harmony with the changes in composition of the milk; (2) it is in agreement with the hydrogen ion concentration shown by normal milk (pH 6.50 to 6.60), and that shown by blood-serum (about pH 7.60); (3) it harmonizes with the variation found by us in the CO_2 content of milk, normal milk containing about 10 per cent by volume and blood-serum, 65 per cent; and (4) it is in agreement with the increasing number of leucocytes found in the less acid milks.

Further proof of the presence of blood-serum or lymph in most of the abnormal milks (pH 6.90 to 7.20) examined by us is the existence of fibrin in such samples, as shown by Doane's method.[18]

Another method of proof was undertaken, which was to ascertain if glucose is present in the abnormal milks under discussion. If serum passes unchanged into milk, glucose should be present in such abnormal samples in appreciable amount. We were, however, unable in any case to find the slightest trace of glucose in these abnormal milks. If glucose is absent from the serum present in these milks, the glucose in the blood-serum must be changed into another compound by some agent present in the milk in the udder, which might be udder cells or some enzyme. The question calls for further investigation.

The method used by us for the detection of glucose is the following: Proteins are precipitated by 70 per cent alcohol and the filtrate evaporated to dryness. This residue is extracted first with ether and then with hot 95 per cent alcohol. The alcoholic extract is evaporated to dryness and the residue again extracted and the extract evaporated to dryness, after which the residue is extracted with a small amount of ether. This residue is used for the osazone test. Glucose added to milk can be recovered easily by this method.

SUMMARY.

1. The object of the investigation was the study of the extent and causes of the variation of the hydrogen ion concentration in freshly-drawn cow's milk.

2. In the case of over 300 samples of fresh milk, the pH value varied from 6.50 to 7.20, being under 6.76 in 80 per cent of the samples.

3. In the case of the milk from 20 cows, it was found that the pH value of the milk from different quarters of the udder varied greatly but in most cases the variations were not large.

4. The pH value is found to vary with the composition of the milk. In general, with a decrease of acidity, there is a marked tendency toward a decrease in specific gravity, and in percentage of fat, total solids, solids-not-fat, casein, and lactose, but an increase in proteins other than casein and in ash and chlorine.

[18] Doane, C. F., *Maryland Agric. Exp. Station, Bull. 102*, 1905.

5. These changes in composition are such as would be expected in case blood-serum or lymph were added to normal fresh milk. Abnormal conditions in the udder might cause such addition.

6. Examination of milks of abnormally low acidity, having a pH value above 6.80, indicates that the reaction is accompanied by the presence of large numbers of leucocytes, though the reaction in such cases may be neutralized by the presence of large numbers of acid-producing streptococci.

7. While the belief in the presence of blood-serum or lymph in such milk is supported by several considerations, a careful test for glucose proved negative.

A METHOD FOR THE PRELIMINARY DETECTION OF ABNORMAL MILK BASED ON THE HYDROGEN ION CONCENTRATION.

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(Received for publication, October 8, 1919.)

INTRODUCTION.

In the official inspection of market milk, the primary object is to detect samples that are abnormal in composition as well as in sanitary character. The full examination of a large number of samples for the purpose of detecting an occasional abnormal one involves a relatively large amount of inefficiency in attaining the object. Attempts have been made to minimize the labor of inspection by using some quick and simple method which would serve the purpose of enabling one to detect suspicious samples; and only those samples which showed some evidence of abnormality by such preliminary test would be selected for further detailed examination in the laboratory, in order to confirm or disprove the suspicion. In examining market milk for the purpose of quickly identifying abnormal samples, inspectors have been limited in their methods to the use of the hydrometer or lactometer, except that in some cases the senses of smell, taste, and sight could also be employed to advantage. The determination of the specific gravity of milk has found its chief use in enabling one to select samples which appear to give evidence of being watered or skimmed. It has been repeatedly shown that specific gravity as a basis for accurate judgment in identifying abnormal milks may be wholly misleading. The need of a more comprehensive and reliable method has long been realized.

The Relation of Hydrogen Ion Concentration to Normal and Abnormal Milks.

In making a study of the hydrogen ion concentration of freshly-drawn milks, normal and abnormal, and also of normal milks subjected to various conditions of change, it was found that the hydrogen ion concentration is very sensitive to certain conditions, among which are (1) production of acid by bacteria, (2) the addition of formaldehyde solution, (3) the addition of acids, (4) heating above a certain temperature, (5) abnormal or diseased milks, (6) addition of water, (7) addition of alkali or alkaline salts, (8) removal of fat. The first four conditions increase the hydrogen ion concentration, that is, render the reaction of the milk more acid than normal, while the other conditions render the reaction of the milk less acid than normal.

It occurred to us that, if it were possible to obtain an indicator having a neutral point near that of normal milk and yet showing an appreciable color in normal milk, which would be sufficiently sensitive to show observable change of color with slight change of hydrogen ion concentration, such an indicator might find application as the basis of a method to be used for the purpose of quickly indicating the probability of normality or abnormality in a milk. The first suggestion of an indicator meeting these conditions came to us in connection with the work published by Clark and Lubs[1] on "A substitute for litmus for use in milk cultures." They made use of a dye, known as dibrom-ortho-cresol-sulfon-phthalein, the name being shortened for convenience to "brom-cresol purple." This dye was found by them to possess properties which make it a reliable and brilliant indicator for the colorimetric determination of hydrogen ion concentration in milk.[2]

We first made use of brom-cresol purple in testing its applicability to the detection of increased acidity in milk when formed by bacterial action, and found that it is extremely sensitive in comparison with phenolphthalein, which is the indicator in common use in titration for the determination of the degree of acidity

[1] Clark, W. M., and Lubs, H. A., *J. Agric. Research*, 1917, x, 105.

[2] This dye can be purchased from Hynson, Wescott, and Dunning, Baltimore, Md. In ordering this dye the full name should be used.

in milk. Further extension of the use of brom-cresol purple demonstrated its practicability in detecting other conditions, especially those mentioned above.

OUTLINE OF PROPOSED METHOD.

Before describing the detailed operation of the method, we will give a brief statement, outlining its main features. The use of brom-cresol purple in this application to the preliminary detection of abnormal milks consists in adding to one drop of a saturated water solution of the dye 3 cc. of milk and then observing the color. In the case of average milks that are normal in character, such, for example, as good market milk, the color is very uniform, being a bluish-gray. In the case of a milk giving a color differing appreciably from this, there is ground for suspicion that it is not normal. The color given by different milks may be lighter or darker, ranging from a bright yellow at one extreme to a deep blue at the other. *The color is made lighter by acids, acid salts, formaldehyde solution, and also by heating above the usual point of pasteurization. The color becomes deeper blue in the case of milk from diseased udders, watered milk, skimmed milk, and milk containing added alkali or an alkaline salt.*

If a preliminary test with brom-cresol purple gives a color lighter than in the case of normal milk, then a sample can be taken by the inspector to be used in making a further detailed examination in the laboratory for acidity, formaldehyde, and overheating. If the color is darker than normal, then a sample is taken to ascertain whether the abnormality is due to the addition of water, alkaline salts, removal of fat, or to the presence of milk from a diseased udder.

Attention should be called here to some conditions which modify the characteristic color given by brom-cresol purple solution with average normal milk.

1. Effect of High Percentage of Milk-Fat.—The presence of extra fat, as in the case of rich milks (5 per cent or more) gives an appreciably lighter color than in the case of the ordinary market milks containing 3 to 4 per cent of milk-fat.

2. Effect of High-Colored Milk.—In the case of milks produced by cows at fresh pasture, the milk has a decided yellow color which modifies the color reaction with brom-cresol purple.

3. Effect of the Removal of Milk-Fat.—When fat is removed from milk, the resulting skim-milk gives with brom-cresol purple a darker color than does the same milk before the removal of fat.

These differences in color are due to the fact the fat-globules do not give the same reaction color as normal milk with brom-cresol purple and they thus modify by their presence the color of the indicator in the milk. The fat dissolves some of the dye which always appears yellow in the fat. The effect of milk-fat upon the reaction color can be readily observed if one notices the color at once after mixing the milk and the solution of brom-cresol purple and then again after the fat-globules have risen to form a cream layer at the upper surface. It will be seen that the color is lighter at the start than it is after the cream has risen and, further, that the cream layer shows little or no color. The difference is more marked with increase of milk-fat.

OPERATION OF METHOD.

1. Preparation of Indicator.

Brom-cresol purple is ground to a fine powder and dissolved in distilled water to saturation, about 0.1 gm. being used for 100 cc. of water. Saturation can be hastened by heating the mixture on a water bath, then cooling to room temperature, and filtering. The saturated solution contains about 0.09 per cent of the dye.

2. Apparatus.

The only apparatus required is the following: a burette, test-tubes, a pipette, and a test-tube holder.

The burette is used for the purpose of measuring the indicator. The delivery is so controlled that each drop measures 0.05 cc.

The test-tubes which we have found most convenient for use in making the test are flat-bottomed specimen tubes made of Pyrex glass, holding about 8 cc. They are about 4 inches long and $\frac{1}{2}$ inch in diameter. It is essential that all the tubes used should be uniform in color and in thickness of walls.

We have found it convenient to provide a special holder for these tubes, making it easy to compare the color by arranging the tubes in a line side by side in close contact without concealing any portion of the milk column.

Ordinary 3 cc. pipettes are used for measuring the milk to be used.

3. Performing the Operation.

The different steps in carrying out the details of the method consist of (*a*) measuring the indicator, (*b*) measuring the milk, (*c*) observation of the color, and (*d*) interpretation of the results.

(*a*) *Measuring the Indicator.*—The test-tubes are placed in the holder with the open end up. The burette is filled with the solution of brom-cresol purple, and the stop-cock so adjusted that it delivers drops measuring 0.05 cc. at the rate of about one drop in 2 seconds. The test-tubes are placed under the burette tip, one by one in turn, exactly one drop being allowed for each tube. The delivery is so controlled that each drop falls free from the burette tip into the test-tube without touching the walls of the tube before the drop separates from the burette tip. This procedure enables one to deliver the same amount of indicator into each test-tube with rapidity. There are two advantages in using only one drop of indicator. The first is the minimum dilution of the added milk and the second is that the tubes can be carried about without danger of losing the indicator.

(*b*) *Measuring the Milk Sample.*—The milk is added to each test-tube with a 3 cc. pipette and is thoroughly mixed with the indicator, which may be conveniently done either by shaking the tube or by drawing the mixture of milk and indicator into the pipette and allowing it to flow back into the test-tube. In our experience the proportion of 3 cc. of milk and one drop of brom-cresol purple solution enables one to observe the shades of color to best advantage in most cases, but in some cases we have obtained somewhat better results in observing color changes with 5 cc. of milk for one drop of indicator.

(*c*) *Observation of Color Change.*—The ability to distinguish shades of color in the change of reaction in milk is the chief point of difficulty and, therefore, the observation of shades of color

constitutes the main source of weakness in its application. The method cannot be used successfully by one whose eye is lacking in appreciation of different shades of color to such an extent that training does not enable one to overcome such deficiency. However, in our experience, any person with normal sensitiveness to color changes can acquire the ability to observe such changes as take place in milk treated with brom-cresol purple solution with an accuracy which will make the application of the method useful. The fundamental difficulty lies in the lack of a fixed color standard which is applicable under all conditions as a basis of comparison. In the examination of market milks, it usually suffices to assume that a large proportion of the samples is normal in reaction and that, therefore, in a collection of numerous samples, those which give the same color with the solution of brom-cresol purple are generally normal, while those samples which are lighter or darker are open to the suspicion of being abnormal in some respect and should be further examined by supplementary methods. It will be well usually, however, especially for those who are unaccustomed to the use of the method, to prepare a series of known standardized colors to be used as a basis of comparison in observing the reaction of unknown milks to which the test is applied.

Preparation of the Color Standard.—The prepared color standard represents approximately certain ranges of hydrogen ion concentration. Briefly stated, the preparation of such a standard consists in adding increasing amounts of standard alkali to a mixture of normal milk and brom-cresol purple solution. The preparation of the series of standard colors to be used for comparison is carried out in the following manner.

(*1st*) *Selection of Milk.*—The milk to be used in the preparation of the color standard should meet two requirements. First, it should have approximately the same general composition as that of the milks to be examined; and, second, it should have a normal reaction. In respect to composition, usually any normal market milk containing between 3 and 4 per cent of fat will be satisfactory in the inspection of market milks. When milks containing over 4.5 per cent of fat are to be tested, it is well to use for the color standard a milk containing about the same percentage of fat. In the case of partially skimmed milks, milk with less

than the normal percentage of fat should be used. The differences in color caused by the presence of varying percentages of fat can be largely overcome, when necessary, by removing the cream with a centrifuge from all the milks to be examined, using skim-milk in the preparation of the color standard. However, it should be stated that the color given by skim-milk obtained from milk rich in fat may be slightly different from that given by skim-milk obtained from milk poor in fat. After one has had some experience in studying the effect of different milks upon the solution of brom-cresol purple, it will be found that the matter of the selection of the milk for making a color standard is simpler than it might appear from the foregoing statements.

In respect to the reaction, the milk to be used in preparing the color standard should not have an acidity in excess of an equivalent of 18 cc. of 0.1 N NaOH per 100 cc. of milk. The determination is made by titrating 10 cc. of milk without dilution with the alkali, using 0.5 cc. of a neutralized, alcoholic solution of phenolphthalein for indicator. The reaction of the milk to be used for the color standard can be further tested by adding brom-cresol purple solution and comparing the resulting color with that given by this indicator with samples of milk of known normal character. If the reaction is uniform with that of the known normal milks, the milk can be satisfactorily used for the color standard. Usually a milk having an acidity equivalent to 1.8 cc. of 0.1 N NaOH per 10 cc. of milk is found to be satisfactory.

(*2nd*) *Preparation of Standard Color Series.*—Measure eight portions of milk of 10 cc. each into separate test-tubes and to each portion add the amount of 0.1 N NaOH indicated below.

Test-tube No.	1	2	3	4	5	6	7	8
No. of drops of 0.1 N NaOH	0	2	4	6	8	10	12	14

The alkali is added to the tubes from a burette, using the same precautions in regard to uniform size of drops and their delivery as have been already given for measuring the brom-cresol purple solution. The alkali and milk are thoroughly mixed. Of the mixture in each tube take 3 cc. and one drop of brom-cresol purple solution, making a series of eight mixtures contained in the kind of test-tubes previously described. These give a range

of colors to be used as a standard for comparison in testing unknown milks. The reaction color in each tube corresponds approximately to the following pH values.

No. in series	1	2	3	4	5	6	7	8
cc. of 0.1 N NaOH used	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
pH value	6.5 to 6.6	6.6 to 6.67	6.67 to 6.75	6.75 to 6.82	6.82 to 6.90	6.90 to 6.98	6.98 to 7.05	7.05 to 7.13
Symbol for reaction color	N	N−1	N−2	N−3	N−4	N−5	N−6	N−7

As a matter of convenience in tabulating results, we append a series of symbols to indicate the pH values, N standing for normal reaction and N followed by the minus sign and figures ranging from 1 to 7, indicating decreased acidity corresponding to increasing pH values.

It should be emphasized here that this method gives the reaction only approximately and the accuracy of the results may be interfered with by various conditions apart from the true reaction. Of such interfering factors, one which is especially influential in affecting the color is the degree of opaqueness. Milks of relatively low acidity tend to be less opaque than those of normal acidity. As a result the surface color observed is reflected from a greater depth and with a corresponding increase in the intensity of the color in the presence of brom-cresol purple solution, thus indicating a reaction less acid than that indicated by the standard. When necessary, this source of interference can be obviated by so diluting the standard series with water after the addition of alkali and before the addition of the brom-cresol purple solution that the milk to be used as a standard will resemble in its appearance of opaqueness that of the unknown milk. The milk thus diluted is then used in making up a special standard series by addition of indicator. While the addition of water changes the reaction of the standard somewhat, the test of the reaction by the color is made more accurate than if the standard is undiluted.

Another factor which may render less accurate the use of the standard in determining the reaction of unknown milks is the initial reaction of the milk used for the standard. If, for example, the initial reaction is pH 6.5 in one case and pH 6.6 in another,

there would be a difference of 0.1 pH in applying the standard to the reaction of unknown milks.

(*d*) *Interpretation of Color.*—In examining the samples of milk in the test-tubes after addition of the milk to the brom-cresol purple solution, one makes comparison with the prepared color standard; or, in the absence of such a standard, one selects those samples which differ in color markedly from those samples which, according to experienced observation, appear normal, either before or after the separation of the cream. Such samples as appear to be abnormal by showing a deeper blue shade of color, indicating decreased acidity, are open to the suspicion of being watered, or skimmed, or treated with alkaline salts, or containing excessive numbers of leucocytes as in milk from diseased udders. Which of these suspicions is justified can be ascertained by the determination (1) of the freezing-point, (2) of the percentage of milk-fat or the ratio of fat to proteins, (3) of the specific gravity, (4) of the total solids, (5) of the presence of alkaline salts, especially sodium bicarbonate and borax, (6) of the numbers of leucocytes by direct microscopic examination by Breed's method,[3] and (7) of CO_2[4] by Van Slyke's method modified by us for use in connection with milk.

In the case of samples showing a color lighter than normal with the brom-cresol purple solution, indicating an abnormal degree of acidity, there is awakened the suspicion of bacterial acid production, the presence of formaldehyde, overheating, or the presence of added acid salts; or the lighter color may be due to a high percentage of milk-fat. Which of these indications is correct is determined as follows: A direct count of the number of bacteria[3] is often sufficient. If this fails to show the presence of excessive numbers of bacteria, then a test should be made for the presence of formaldehyde, and, if this is not present, the percentage of milk-fat is determined; and, further, in order to see if the light color is due to overheating, the determination of carbon dioxide should be made and Storch's test may also be applied.

[3] Breed, R. S., *New York Agric. Exp. Station, Techn. Bull. 49*, 1916.
[4] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1919, xl, 337.

RESULTS OF APPLICATION OF METHOD.

Relation between the Reaction of Milk and the Presence of Added Water and of Garget.

We have made application of this preliminary test extensively in the case of market milk and especially in connection with watered milk and milks containing garget and also milks with a high bacterial content.

Of 570 samples of market milk to which the test was applied, 64 samples, of which 52 showed decreased acidity, were selected by the brom-cresol purple test for more detailed examination in relation to the presence of added water and high leucocyte content. The results of the work are summarized in Table I. Each sample of market milk represented a single can of milk as delivered at the milk station by the producer. Milk from the same producer was sampled on several different days in those cases in which there was cause for suspecting the milk to be abnormal. Most of the samples giving a normal color reaction (N) were selected from the milk of producers whose milk appeared on previous inspection to show signs of being watered but which gave a normal reaction at the time of this sampling. There are given in addition results in the case of a few samples (Nos. 23 to 28) of milk obtained from different quarters of the udders of cows, some of which were known to have udder infection, and there are also three samples (No. 6) of normal mixed milk from our Station herd.

An examination of the results contained in Table I leads to the following summarized statements:

1. Of the selected 64 samples of market milk, 52 show a color reaction less acid than normal and 12, a normal reaction.

2. In all the samples giving a normal color reaction, the depression of the freezing-point varies between -0.54 and $-0.57°$. In the 52 samples of market milk showing a reaction less acid than normal, constituting nearly 10 per cent of all the samples examined by the color test, 39 samples show a depression of the freezing-point varying from -0.468 to $-0.538°$, thus indicating the presence of added water. In 18 samples giving N-1 color reaction (pH 6.6 to 6.67), 10 samples show watering by the

TABLE I.

Results of Examination of Market Milk by Brom-cresol Purple Test.

Herd No.	Reaction.	Depression of freezing-point.	Garget.	Herd No.	Reaction.	Depression of freezing-point.	Garget.
		degree				*degree*	
1	N	0.569	0	13	N-2	0.538	0
2	"	0.558	0	13-a	"	0.509	0
3	"	0.558	0	14	"	0.477	0
4	"	0.560	0	14-a	N	0.548	0
4-a	"	0.560	0	14-b	N-2	0.530	0
6	"	0.548	0	14-c	"	0.512	0
5	"	0.552	0	14-d	"	0.490	0
6-a	"	0.555	0	14-e	"	0.523	0
6-b	"	0.562	0	14-f	"	0.503	0
7	N-1	0.536	Present.	14-g	"	0.513	0
7-a	N	0.546	0	14-h	"	0.520	0
7-b	N-1	0.546	Present.	14-i	"	0.525	0
7-c	"	0.550	"	14-j	"	0.500	0
7-d	"	0.536	"	14-k	"	0.530	0
7-e	N	0.558	0	15	"	0.518	0
7-f	N-1	0.552	Present.	15-a	"	0.536	Present.
7-g	"	0.549	"	15-b	"	0.543	"
7-h	"	0.549	0	15-c	N-1	0.518	0
7-i	N	0.553	0	16	N-2	0.523	0
8	N-1	0.528	0	16-a	"	0.506	0
8-a	"	0.547	0	16-b	N-1	0.512	0
8-b	N-2	0.530	0	16-c	N	0.556	0
8-c	N-1	0.545	0	16-d	N-2	0.496	0
8-d	"	0.538	0	17	"	0.494	0
8-e	N-2	0.541	0	18	N-3	0.519	0
8-f	N-1	0.563	Present.	19	N-2	0.530	0
8-g	N-2	0.540	0	20	N-1	0.491	0
9	"	0.518	0	21	"	0.531	0
9-a	"	0.531	0	22	N-2	0.540	0
9-b	"	0.512	0	22-a	"	0.540	0
9-c	N-1	0.530	0				
10	N-2	0.500	0	23	N	0.558	0
10-a	N-1	0.512	0	24	"	0.558	0
10-b	N	0.558	0	25	N-4	0.540	Present.
10-c	N-3	0.470	0	26	"	0.563	"
11	"	0.468	0	27	N-5	0.558	"
12	"	0.519	0	28	"	0.548	"

freezing-point determination, while 8 do not. Of these 8 samples, the decreased acidity is due to the presence of garget in 5 cases. The other 3 cases are on the border line as shown by the freezing-point depression. In 30 samples showing N-2 color reaction (pH 6.67 to 6.75), 25 contain added water according to the freezing-point, while 5 are just on the border line, showing a freezing-point depression varying from $-0.54°$ (in 3 cases) to $-0.543°$. In the case of 4 samples (10-c, 11, 12, 18), giving a color reaction of N-3 (pH 6.75 to 6.82), all showed the pres-

TABLE II.

Results of Examination by Herds.

Herd No.	No. of times examined.	No. of samples with subnormal reaction.	No. of samples watered.	No. of samples with garget.
7	10	7	0	6
8	8	8	3*	1
9	4	4	4	0
10	4	3	3	0
11	1	1	1	0
12	1	1	1	0
13	2	2	2	0
14	12	11	11	0
15	4	4	3†	1
16	5	4	4	0
17	1	1	1	0
18	1	1	1	0
19	1	1	1	0
20	1	1	1	0
21	1	1	1	0
22	2	2	0	2

* 2 others doubtful.

† 1 other doubtful.

ence of added water by the freezing-point. In the case of 2 samples (25, 26), having a color reaction of N-4 (pH 6.82 to 6.90) and of 2 samples (27, 28) with a reaction of N-5 (pH 6.90 to 6.98), the decreased acidity was due in every case to garget, the determination of the freezing-point showing the milks to be entirely normal in water content. For further details regarding the reaction of milk to udder infection see page 351.

3. The number of dairies furnishing the 570 samples of milk examined was 46. In the case of 16 dairies, the milks showed a

subnormal or decreased acid reaction by the brom-cresol purple test. Three-fourths of these subnormal samples came from a few herds. It was found that in one of these the milk was being watered regularly and some of the milk from the other herds gave evidence of severe mastitis. Table II shows the number of milks of subnormal reaction in the case of the 16 herds and indicates also the total number of examinations and the number of times the samples were found watered and, in addition, the cases where garget was present.

It is seen that in the case of the 16 herds, 58 examinations of milk were made; in 52 samples, the reaction was found to be subnormal or of decreased acidity. In these 52 cases, 37 showed clear evidence of watering by the depression of the freezing-point and 3 others were so close to the border line as to be open to a strong suspicion of being watered. There were three herds in which persistent addition of water was shown.

4. The 6 samples, 23–28, were drawn from the udders under our direct supervision. Four of these showed a subnormal reaction, owing to the presence of garget, and the freezing-point test shows that the percentage of water is not excessive. A complete chemical analysis of these four samples would undoubtedly show an abnormal composition according to our work (page 350).

Relation Between the Reaction of Milk and the Bacterial Content.

Milks showing a reaction above normal acidity, as indicated by giving with brom-cresol purple solution a lighter color, were examined for their bacterial content. Of the 570 samples examined, 16 gave a lighter color than normal, of which 11 were found by Miss Mildred C. Davis, the City Bacteriologist, to contain over 10,000,000 bacteria per cc. by the direct-count method.[3] In the case of two of the other samples, the light color was due to high milk-fat content, bacteria not being present in large numbers.

A further study was made at the Laboratories of the Department of Health of New York City through the courtesy of the Director, Dr. Wm. H. Park. Of the 11 samples found showing a light color with brom-cresol purple solution, four contained

over 1,000,000 bacteria per cc. by the plate-count method and six showed high percentage of milk-fat with low bacterial content.

SUGGESTIONS.

It has been stated already that the main source of weakness in the application of this method is the observation of the shade of color given by the sample of milk with brom-cresol purple solution. It is, therefore, important that, before one attempts to use the method in practical application, some special work be done in a study of the shades of color of the brom-cresol purple solution in milk under a great variety of conditions. For example, taking some fresh normal milk of average composition, that is, with 3 to 4 per cent of milk-fat, a portion is treated with brom-cresol purple solution in the manner described (page 361), and then other portions are treated by the addition of definite amounts of 0.1 N alkali, just as in the preparation of the standard color series (page 363), and other portions by 0.1 N lactic acid, while other portions are diluted with definite amounts of water, and others are skimmed, and others have cream added to them. Also the results of the action of definite amounts of formaldehyde added to portions of the milk should be studied; and also the effects of the addition of varying amounts of sodium bicarbonate, borax, etc. Portions of milk heated to various temperatures are similarly studied. A similar complete study should be made with different samples of normal milk until one is able to distinguish different shades of color so far as they have a meaning in practical applications of the test.

It should be emphasized here again that the application of the brom-cresol purple test is not to be regarded as final but only as preliminary and suggestive. Its chief value is to be found in the fact that, when properly used, it will greatly minimize the work involved in official milk inspection, because it will point in most cases directly to the milks that are abnormal and, therefore, indicate which samples need further detailed work to confirm or disprove the suspicion aroused by the result of the preliminary test.

SUMMARY.

The method described as a means for the preliminary detection of abnormal milks is based upon the use of the dye, dibrom-ortho-cresol-sulfon-phthalein, the name being abbreviated to brom-cresol purple. One drop of a saturated water solution is mixed with 3 cc. of milk and the color is observed. Normal fresh milk gives a grayish-blue color. The production of a darker or lighter color serves to awaken suspicion in regard to the normal character of the milk. The color is made lighter by acids, formaldehyde, and also by heating above the usual point of pasteurization. The color becomes deeper blue in the case of milk from diseased udders, watered milk, skimmed milk, and milk containing added alkaline salts. In the inspection of milk, a sample is taken for further detailed examination in the laboratory if the color is sufficiently lighter or darker than normal to indicate the probability of some abnormal condition.

The method has been applied and results are reported for 570 samples of market milk. Watered milk was detected and also milk containing excessive numbers of leucocytes. A standard of colors can be prepared by which comparison can be made and conclusions more easily reached as to the normality or abnormality of the samples examined.

A METHOD FOR THE DETERMINATION OF THE KEEPING QUALITY OF MILK.

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(Received for publication, October 8, 1919.)

INTRODUCTION.

Keeping quality or keeping power is an expression used to indicate the length of time milk remains sweet and otherwise palatable and suitable for direct consumption. This is obviously an important factor in estimating the commercial value of market milk, since milk that is sour or otherwise unpalatable is comparatively valueless for direct use, however rich it may be in fat and other solids.

Various methods have been proposed for measuring the keeping quality of milk but these have been found unsatisfactory in actual practice. Therefore, a method which can be utilized to furnish consistent results in measuring, even though only approximately, the keeping quality of different milks is needed.

Proposed Method.

We have found that the brom-cresol purple test (see page 359) can, with simple modifications in technique, be applied to the measurement of certain factors affecting the keeping power of milk. In applying the test for this purpose, the test-tubes and pipettes must be sterilized before use and the milk in the test-tubes must be incubated for a stated length of time at a definite temperature. Examination of the milk after incubation furnishes evidence in respect to the keeping power of milk as shown by one or more of several possible changes that may take place in the milk. Such changes can be divided into two classes, first, those affecting the color of brom-cresol purple, which show a

change of reaction in the milk due to the production of acid or less often to the formation of alkali salts; and, second, other accompanying or succeeding changes, such as curdling of the milk due to coagulation of casein, digestion of casein, changes in the character of the coagulated or curdled milk, production of gas, and the development of abnormal odor and taste.

Non-Germicidal Effect of Brom-Cresol Purple Solution.

In order to be of value as a means of measuring the keeping quality of milk by the reaction, it is essential that the brom-cresol purple solution should not, under the conditions used, show any germicidal effect sufficient to interfere with the growth of bacteria in milk. In order to test this fundamental requirement, pure cultures of *Bacterium lactis acidi* were added to freshly pasteurized skim-milk; one portion of this was treated with brom-cresol purple solution and both portions were incubated at 20°C. At intervals the brom-cresol purple test was applied to samples taken from the incubated portion of milk containing none of the indicator, and comparison was made with the portion to which brom-cresol purple had been added at the start. Also samples of the two portions of milk were titrated with alkali. These tests were made many times with different milks, but in no case was there observable any difference in behavior. The same tests were also applied in numerous cases to two portions of a milk undergoing the process of natural souring, using both unheated and pasteurized milk, without showing any difference. The results all go to show that the brom-cresol purple solution has no germicidal effect under the conditions used. However, it is advisable to take precaution to use for this test only brom-cresol purple that is wholly free from the odor of phenol or cresol, as suggested by Clark and Lubs.[1] In our experience it is not difficult to obtain this.

Production of Acid.

In considering the application of the brom-cresol purple test to the measurement of the keeping quality of milk as shown by the formation of acid in milk, we will present the subject under

[1] Clark, W. M., and Lubs, H. A., *J. Agric. Research*, 1917, x, 105.

the two heads, localization of acid production and degrees of acid production.

Localization of Acid Production.—In the natural souring of milk standing undisturbed, the formation of acid rarely proceeds uniformly through the body of the milk but is largely localized, especially in the earlier stages of the process. Acid is usually first formed in appreciable amount at the upper surface next the cream layer, or less often in the layer at the bottom of the container, or it may appear in some cases simultaneously in both the top and bottom layers. Less frequently it may start in the layer of milk next the side walls of the container.

When acid is formed first at the upper surface of the milk, it is probably due to the fact that the organisms are enmeshed and carried upward with the rising fat-globules and are thus concentrated in the upper layer. The bacteria left in the body of the milk after the rising of the fat-globules would tend, under the downward pulling effect of gravity, to settle at the bottom of the container. Generally, the number carried up is apparently greater than that carried down. Such a concentration of bacteria in the top or bottom layer of the milk would have the effect of making the brom-cresol purple test more sensitive as a result of more rapid formation of acid. The effect of acid development is more commonly shown first in the upper layer, though sometimes in the lower or less often in the side layer. But whether it starts at the top or bottom or side, the process of acid production works from the starting area or areas through the main body of the milk.

Degrees of Acidity.—It would be desirable, if it were possible, to distinguish different degrees of increasing acidity by preparing a color standard representing different values of hydrogen ion concentration, similar to the method described on page 363 for determining the approximate hydrogen ion concentration of milk when its acidity is less than that of normal milk. This is impossible for several reasons and especially because, as pointed out above, the production of acid is localized and not distributed uniformly through the body of the milk. However, we have found that it is possible, with some experience, to distinguish readily not less than four degrees or stages of acidity by changes of color, varying from the grayish-blue observed with normal

fresh milk to a pure yellow occurring in milk sufficiently sour to undergo coagulation, which occurs at about pH 4.65. These four stages or degrees of acidity can be distinguished by the following description.

(1) The first stage or beginning of acid production (A_1) is indicated by the first sign of change from the grayish-blue color of normal milk to a lighter shade observable in any portion of the milk. This is most often distinguishable at the upper surface of the milk just under the cream layer, though it may occur at the bottom layer of the milk or less frequently at the side walls of the containing vessel.

(2) The second stage (A_2) shows distinct acid production and is indicated when the milk in a test-tube gives evidence of more extensive and marked change of color than in case of A_1. The main body of the milk, however, still retains a grayish-blue color more or less interspersed with, but predominant over, yellowish or greenish-yellow shades. The prevailing color may be bluish or a dull shade of bluish-green.

(3) The third stage (A_3) shows marked acid production and is indicated when the color of the milk in the test-tube appears greenish to greenish-yellow; the yellow is predominant through the body of the milk, though not complete, but is more or less interspersed with shades intermediate between dull green and yellow.

(4) The fourth stage (A_4) of acid production is easily observable, since the color is a pure, fairly uniform yellow, free from every trace of bluish or greenish tints. The curdling of the milk usually occurs at this stage and is generally, though not always, readily seen.

It can easily be understood that these divisions are somewhat arbitrary and not always capable of sharp separation but they afford a practical basis for differentiating milks, furnishing a test which greatly exceeds in delicacy and ease of application the usual titration methods. With experience in distinguishing shades of color, it is easily possible to carry the division of classes further if desired; but usually the four broad classes described above suffice for most purposes.

Other Changes in Milk.

Changes other than those produced by acid formation can also be observed, and to these attention will be briefly called. Such changes may occur only after somewhat prolonged incubation in the case of good market milks, but they appear more quickly in the case of milks which have been drawn more than 24 hours before incubation, or in the case of milks drawn under unfavorable conditions as to cleanliness and not kept at a sufficiently low temperature. It should be stated here that while these changes have been studied by bacteriologists, it is essential that they be given special attention and further study under the conditions of the proposed test.

1. *Production of alkali* during incubation is shown by decreased acidity and is indicated by increase of depth of the grayish-blue color given with normal milk by the brom-cresol purple solution.

2. *Digestion of casein* is observable just below the cream layer and is indicated by the appearance of a layer of more or less clear solution.

3. *Gas production* is easily observed, indicating the presence of gas-producing organisms. This test is especially valuable in connection with milk to be used for cheese-making.

4. *The contraction or shrinking of the curd* or coagulated casein into a smaller mass is easily seen when it occurs. This is accompanied by the separation of more or less clear whey.

5. *Any abnormal odor or taste* is readily ascertained by any one having well developed senses of smell and taste. Such abnormal conditions have been noticed in our experience only in the case of milks which had shown marked change in reaction as indicated by the color given with brom-cresol purple solution.

RESULTS OF APPLICATION OF THE METHOD.

In applying the brom-cresol purple test to the measurement of acid production in relation to the keeping quality of milk, two separate series of experiments will be presented. In the first series, the samples used were taken from individual cans of milk as delivered by producers at the two collecting stations handling the supply of the city of Geneva. In the second series,

the samples were obtained from the regular milk supply of New York City.

1. The Geneva Samples.—In applying our method to 389 samples obtained in Geneva, we have had the cooperation of the city bacteriologist, Miss Mildred C. Davis, who classified the samples into groups by microscopical examination, using the direct-counting method.[2]

The results are summarized in Table I.

In interpreting the results of the microscopical examination with reference to the fitness or keeping quality of milk for domestic use, milks in Class I are regarded as excellent, in Class II as satisfactory, in Class III as unsatisfactory, and in Class IV as

TABLE I.

Comparison of Results of Brom-Cresol Purple Test with Classification by Microscopical Examination.

Class.	No. of individual bacteria per cc. of milk.	No. of samples examined.	No. of samples changing color.	No. of samples not changing color.	Milk showing good keeping quality.	Milk showing poor keeping quality.
					per cent	*per cent*
I	Below 350,000	283	41	242	85.5	14.5
II	Between 350,000 and 1,000,000	21	6	15	71.5	28.5
III	" 1,000,000 and 10,000,000	52	33	19	36.5	63.5
IV	Over 10,000,000	33	27	6	18.0	82.0

very unsatisfactory. While there is a general correspondence between the results obtained by the microscopical examination and by the brom-cresol purple test, the agreement is not complete. In Class I, representing milk of excellent quality by microscopical examination, 242 samples out of 283 show no change by the brom-cresol purple solution, thus confirming the results of the microscopical examination; but 41 samples out of the 283, or 14.5 per cent, show sufficient increase of acidity to be detected by the brom-cresol purple solution. In Class II, of the 21 samples graded as satisfactory by microscopic examination, 6 samples, or 28.5 per cent, show increase of acidity with brom-cresol purple solution. In Class III, 52 samples are graded as unsatisfactory

[2] Breed, R. S., *New York Agric. Exp. Station, Techn. Bull. 49*, 1916.

by the microscopic method, while 19 samples, or 36.5 per cent, fail to show increased acidity. In Class IV, 33 samples are graded as very unsatisfactory by the microscopical method, but of these there are 6, or 18.0 per cent, which show no increase of acidity. These observed differences of interpretation in the application of these two methods to the determination of keeping quality in milk are what might be expected under the conditions and are easily explained. The brom-cresol purple test is here applied to detect increase of acidity while the microscopical examination includes all kinds of bacteria and not merely those capable of producing acid. It is obvious that in the case of bacteria producing no acid or only very small amounts under the conditions of the test, the brom-cresol purple test would not be expected to apply as it does in the case of marked acid producers.

2. The New York City Samples.—These samples were obtained in the regular inspection work of the city milk supply, through the courtesy of Dr. Wm. H. Park, Director of the Laboratories of the Department of Health. We are indebted also to Dr. Hazel Hatfield for the work done in making the bacteriological examinations of the samples. There were examined 220 samples of unheated or raw milk and 186 of pasteurized milk. The bacteriological examination was made by the official plate method after incubation at 37°C. The tests with brom-cresol purple solution were made in all cases on samples incubated at 18°C. for 24 hours. This temperature is higher than that found in efficient household refrigerators, but it may be regarded as representing approximately the average temperature at which milk is kept after delivery to the consumer.

The division into classes on the basis of bacterial content has been carried farther than in case of the Geneva milks, providing fifteen different divisions as shown in Table II.

The results with the raw milks examined in New York City show, in general, that when the numbers of bacteria increase, there is an increase in the percentage of samples showing increase of acidity. Milks with high bacterial content usually show poor keeping quality by the brom-cresol purple test, while those containing small numbers of bacteria generally show good keeping quality by the color test. However, some milks with fairly high

TABLE II.

Results of Examination of New York City Milks.

Class.	R = raw P = pasteurized.	No. of colonies developed per cc. of milk.	No. of samples.	No. of samples showing change of reaction.	No. of samples not showing change of reaction.	Milk classed as poor by test.	Milk classed as good by test.
						per cent	*per cent*
I	R	Below 1,000	0	0	0	0	—
II	"	Between 1,000 and 5,000	4	0	4	0	100
III	"	" 5,000 " 10,000	6	0	6	0	100
IV	"	" 10,000 " 20,000	4	3	1	75	25
V	"	" 20,000 " 30,000	10	5	5	50	50
VI	"	" 30,000 " 60,000	17	14	3	82.4	17.6
VII	"	" 60,000 " 100,000	23	22	1	95.7	4.3
VIII	"	" 100,000 " 150,000	25	20	5	80	20
IX	"	" 150,000 " 250,000	57	54	3	94.7	5.3
X	"	" 250,000 " 400,000	16	14	2	87.5	12.5
XI	"	" 400,000 " 600,000	16	16	0	100	0
XII	"	" 600,000 " 1,000,000	20	20	0	100	0
XIII	"	" 1,000,000 " 1,500,000	10	9	1	90	10
XIV	"	" 1,500,000 " 3,000,000	7	7	0	100	0
XV	"	Over 3,000,000	5	5	0	100	0
I	P	Below 1,000	4	0	4	0	100
II	"	Between 1,000 and 5,000	16	1	15	6.2	93.8
III	"	" 5,000 " 10,000	9	1	8	11	89
IV	"	" 10,000 " 20,000	32	0	32	0	100
V	"	" 20,000 " 30,000	32	2	30	6.2	93.8
VI	"	" 30,000 " 60,000	28	3	25	10.7	89.3
VII	"	" 60,000 " 100,000	20	5	15	25	75
VIII	"	" 100,000 " 150,000	15	9	6	60	40
IX	"	" 150,000 " 250,000	13	12	1	92.3	7.7
X	"	" 250,000 " 400,000	4	2	2	50	50
XI	"	" 400,000 " 600,000	5	5	0	100	0
XII	"	" 600,000 " 1,000,000	3	3	0	100	0
XIII	"	" 1,000,000 " 1,500,000	5	5	0	100	0
XIV	"	" 1,500,000 " 3,000,000	0				
XV	"	Over 3,000,000	0				

bacterial content show good keeping quality, while some with a low content show poor keeping quality, by the brom-cresol purple test.

With pasteurized milks we obtain similar results, except that a large proportion of the milks with the higher bacterial content show good keeping quality by the brom-cresol purple test.

The results obtained with the New York City milks are not properly comparable with those obtained with the Geneva milks, especially for two reasons. In the first place, the two methods of obtaining the bacterial content, Breed's direct-counting method and the so-called official plate method, do not give results sufficiently comparable for our purpose. In the second place, the New York City milks average probably not less than 24 hours old when the samples are used for laboratory work, while the Geneva samples are not more than 6 to 16 hours old.

In the summary of the results here presented, we do not give the varying degrees of acidity developed on incubation but only the general fact of an increase. Data in greater detail are being collected.

Additional Work.

While some observations have been made on the relation of the other factors to keeping quality, much additional work remains to be done along the following lines: (1) Digestion of casein, (2) production of alkali, (3) production of gas, (4) taste and odor, (5) relation of age of milk to temperature and length of time of incubation.

SUMMARY.

1. Brom-cresol purple can be used to measure approximately and relatively the keeping quality of milk. The test is applied in the manner described in the article preceding (page 357), with the modification that the pipettes and test-tubes used are sterilized before sampling the milk, and, further, the samples of milk in the test-tubes must be incubated a given time at a given temperature (usually 18° to 20° C.). The milk is examined for changes of color at 24 hour intervals. The main factor shown by this test as related to keeping quality is production of acid, but

additional factors to be observed are coagulation of casein, digestion of casein, production of alkali, production of gas, development of abnormal odor and taste.

2. In showing the development of acidity, four stages of progress are distinguishable through change of color, varying from the grayish-blue of brom-cresol purple in normal milk to a final clear yellow, the intermediate stages showing mixtures of color. In comparing this test with the bacterial count, it is found that in general large numbers of bacteria and increase of acidity are in fair correlation.

3. The other factors related to keeping quality, such as digestion, gas, alkali production, and abnormal odor and taste, are readily observable, but frequently not until after 24 hours of incubation.

EXTRACTION AND CONCENTRATION OF THE WATER-SOLUBLE VITAMINE FROM BREWERS' YEAST.*

By THOMAS B. OSBORNE and ALFRED J. WAKEMAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.)

(Received for publication, October 1, 1919.)

In making experiments to determine the nutritive value of individual proteins it is necessary to employ a source of the water-soluble vitamine in the synthetic diet. Heretofore it has been customary to feed some natural product such as protein-free milk, wheat embryo, or yeast to supply the necessary amount of this vitamine. All such products, however, contain relatively considerable quantities of nitrogenous substances, including proteins, hence there is always a question as to whether, or not, these so supplement deficiencies in the protein being tested as to give more or less misleading results. Such criticisms are not entirely unjustified because the nitrogen supplied together with the vitamine may be equal to 10 to 12 per cent of the nitrogen fed. Since some of such nitrogen belongs to protein and most of this to amino-acids identical with those contained in the protein being tested there is little reason to believe that the results of the experimental feeding are seriously affected. Nevertheless there is always a possibility that this may happen and we have had a feeling that some of our results may have been affected to a noticeable extent. A concentrated preparation of the water-soluble vitamine would also be of value for feeding experiments with inorganic salts as well as with individual carbohydrates. With a view to producing such a preparation we have attempted to concentrate the water-soluble vitamine in a fraction of yeast.

We have long known that the water-soluble vitamine is insoluble in absolute alcohol, hence it seemed probable that by

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

fractionally precipitating the aqueous extract with alcohol this might be concentrated in a fraction and thus be obtained together with a comparatively small proportion of the other constituents of the yeast.

The first step towards this end was the preparation of an aqueous extract which should contain as much of the vitamine and as little of the other constituents of the yeast as possible. Almost all investigators who have attempted to separate the water-soluble vitamine from yeast have apparently considered it necessary to bring into solution as much as possible of the contents of the yeast cells. To accomplish this they have generally allowed the yeast to undergo autolysis, whereby a considerable part of the protein, as well as of other constituents of the yeast, is converted into water-soluble products largely of unknown character.

If the protein in the living yeast could be coagulated by boiling water before undergoing any autolytic change, and the water-soluble vitamine be simultaneously extracted, the conditions for a concentration of this vitamine in a fraction of the water extract would be greatly simplified.

EXPERIMENTAL.

ral [illegible] of fresh bottom yeast was obtained directly from a brewery, and immediately diluted with ice water. After centrifuging, the sediment was washed twice more in the same way. The moist, washed yeast weighed 264 gm., equal to 48 gm. dried at 107°. This was stirred gradually into 1 liter of boiling distilled water containing 10 cc. of 1 per cent acetic acid. After boiling for about 2 minutes the solids were separated from the extract with the centrifuge. The residue was washed once by boiling with 0.01 per cent acetic acid and, after centrifuging, the extracts were united and concentrated to 500 cc. This concentrated extract contained 8.14 gm. of solids, equal to 17.1 per cent of the dry yeast, and 0.666 gm. of nitrogen, equal to 14.4 per cent of the original yeast nitrogen, or to 8.18 per cent of the solids of the extract.

Although this extract contained less than one-fifth of the yeast solids it contained nearly all the water-soluble vitamine.

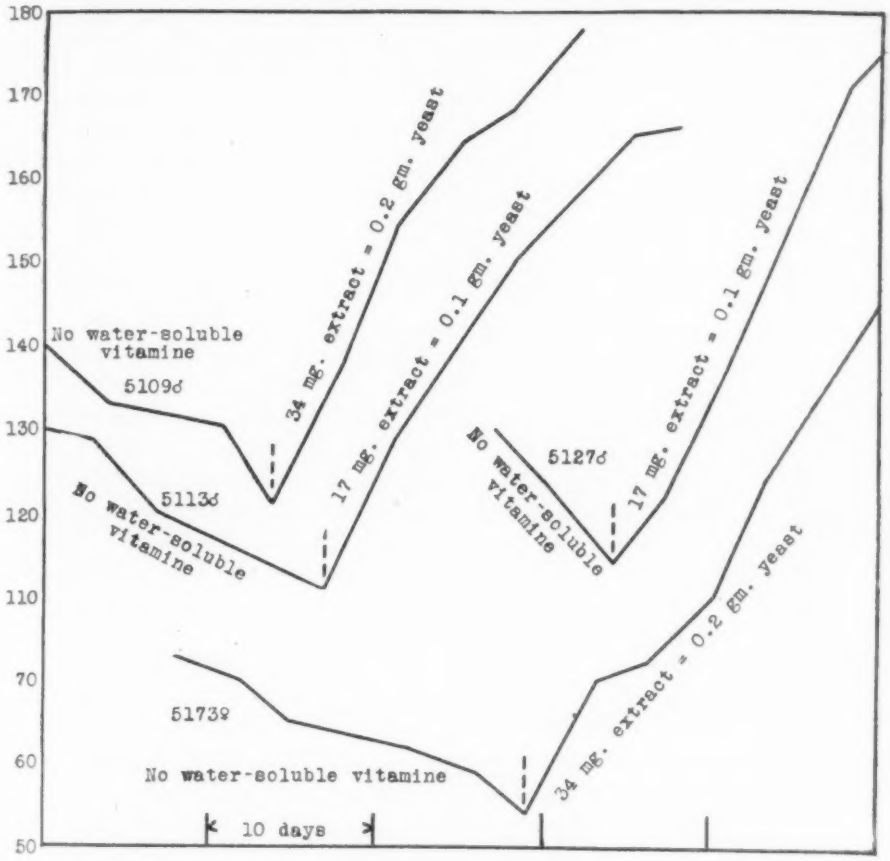

CHART I. Young rats declining on a diet free from water-soluble vitamine recover when the solids extracted from live yeast by boiling water are added in a quantity equal to 0.1 to 0.2 gm. of the original yeast.

Chart I (see Rats 5113 and 5127) shows that daily doses of 17 mg. of the solids of this extract promoted the recovery and rapid growth of young rats declining on a diet free from the water-soluble vitamine, but which in other respects was adequate. When 34 mg. of the yeast extract were fed (see Rats 5109 and 5173) the rate of gain was no greater, hence it appears that the smaller dose supplied as much of the water-soluble vitamine as

these animals needed. These doses were equivalent to only 0.1 and to 0.2 gm. respectively of the original dried yeast. Usually 0.1 gm. of dried yeast is not sufficient to promote such a vigorous gain of weight as these rats showed, hence we conclude that this boiling water extract contained nearly all the water-soluble vitamine of the yeast from which it was derived.

When we found that this aqueous extract was so rich in the water-soluble vitamine, a similar extract from a larger quantity of fresh bottom yeast was subjected to fractional precipitation by alcohol. After washing with ice water, the 4.5 kilos of the moist yeast, equal to 830 gm. dried at 107°, were stirred slowly into 10 liters of boiling water containing 0.01 per cent of acetic acid. After boiling about 5 minutes the extract was readily filtered through folded soft papers. The residue was washed once by boiling with 5 liters of 0.01 per cent acetic acid and the washings, united with the main extract, were concentrated to 2 liters. This contained 140 gm. of solids, equal to 16.9 per cent of the dry yeast, and 12.02 gm. of nitrogen, equal to 13.7 per cent of the original yeast nitrogen, or to 8.6 per cent of the solids of the extract. These are substantially the same proportions as were found in the preceding experiments.

The concentrated extract was then poured into 3 liters of 93 per cent alcohol, making the alcoholic content of the mixture about 52 per cent by weight. The flocculent precipitate, Fraction I, which separated, when washed with 52 per cent alcohol, digested with absolute alcohol, and dried over sulfuric acid, formed a nearly white powder, equal to 35.9 gm. dried at 107°. This fraction formed 4.3 per cent of the dry yeast or 25.6 per cent of the solids of the water extract. It contained 1.72 gm. of nitrogen, equal to 1.9 per cent of the original yeast nitrogen, or to 4.8 per cent of the precipitate. Its ash content was 47.15 per cent.

The filtrate and washings from Fraction I were concentrated to 300 cc. and poured into 1,960 cc. of 93 per cent alcohol, making the alcoholic content of the mixture about 79 per cent by weight. The precipitate, Fraction II, thereby produced was washed once with 79 per cent alcohol, and then twice dissolved in about 100 cc. of water, and reprecipitated by pouring into enough alcohol to make the alcoholic concentration 90 per cent by weight. After

digesting under absolute alcohol and drying over sulfuric acid a light-colored, friable product was obtained equal to 51.8 gm. dried at 107°, or to 37 per cent of the solids of the extract, or to 6.2 per cent of the dried yeast. This contained 7.5 per cent of nitrogen, equal to 4.5 per cent of the nitrogen of the original yeast, and 10.65 per cent of ash.

The united solutions from Fraction II were concentrated to a small volume and poured into enough absolute alcohol to make the alcoholic concentration of the mixture 90 per cent by weight. The precipitate which separated, Fraction III, was dissolved in 100 cc. of water and again precipitated by alcohol at 90 per cent. Since this precipitate was gummy it was again dissolved in about 30 cc. of water and the solution poured into 500 cc. of absolute alcohol. The still gummy precipitate was digested with absolute

TABLE I.

	Solids.	Nitrogen.	Solids.		Nitrogen.		Ash of fraction.
			Of yeast.	Of water extract.	Of fraction.	Of yeast.	
	gm.	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Water extract.....	140.0	12.0	16.9	100.0	8.6	13.72	21.70
Fraction I.....	35.9	1.7	4.3	25.6	4.8	1.94	47.15
" II.....	51.8	3.9	6.2	37.0	7.5	4.45	10.65
" III.....	13.9	1.8	1.6	9.9	13.1	2.06	15.90
" IV.....	36.1	3.9	4.4	25.8	10.8	4.45	15.90

alcohol and dried over sulfuric acid. Fraction III weighed 13.85 gm. dried at 107°, equal to 1.6 per cent of the original dried yeast, or to 9.9 per cent of the solids of the water extract, and contained 1.81 gm. of nitrogen equal to 13.1 per cent of the fraction, or to 2.06 per cent of the yeast nitrogen. Its ash content was 15.9 per cent.

The strong alcoholic solutions from Fraction III were united and concentrated to small volume. Owing to the gummy character of the dissolved solids these were preserved in strong alcohol. This solution contained solids, Fraction IV, equal to 36.1 gm. dried at 107°, equivalent to 4.4 per cent of the original yeast or to 25.8 per cent of the solids of the water extract. It contained 10.83 per cent of nitrogen, equivalent to 4.5 per cent of the yeast nitrogen, and 15.9 per cent of ash. The results of this fractionation are summarized in Table I.

If the water-soluble vitamine was wholly concentrated in any one of these fractions quantities of each equivalent to the original yeast from which it was derived should be as effective as the latter in promoting the recovery of young rats declining on a diet free from this accessory. As 0.2 gm. of the entire yeast has proved to be sufficient for this purpose, and 0.1 gm. usually to be insufficient, these fractions were mixed with starch in such proportion as to make the mixture contain the same percentage of the fraction as did the original yeast. Thus 0.2 gm. of the starch-fraction mixtures contained of Fraction I 8.6 mg., II 12.4 mg., III 3.2 mg., IV 8.8 mg. Since Fraction II formed a larger percentage of the yeast than did any of the other fractions the absolute amount of each of these fractions in the respective starch mixtures was less than that of Fraction II. The feeding experiments to be described consequently do not afford a strict comparison of the efficiency of the fractions relatively to one another. They do, however, enable us to determine in which fraction the greater part of the total water-soluble vitamine of the yeast was concentrated. Charts II, III, and IV show the results of such feeding experiments. Chart II (Rats 5191, 5199, and 5291) shows that no appreciable gain of weight was made when doses of Fraction I equivalent to 0.2 gm. of yeast were fed daily. That this fraction was not wholly free from the water-soluble vitamine is shown by the slight gains made by Rats 5191 and 5291 after doubling the dose. When Rat 5199 was given daily doses of Fraction II corresponding to 0.2 gm. of yeast, it gained weight rapidly. Chart III shows that Rats 5116 and 5289 which received daily doses of Fraction II equivalent to 0.2 gm. of yeast gained weight rapidly. Rat 5116 when given 0.2 gm. of yeast grew no faster. Rat 5198 which received a daily dose of Fraction II equivalent to only 0.1 gm. of yeast made a fairly rapid gain of weight although only 6.2 mg. of the fraction were fed. Chart IV shows that Rats 5054 and 5288, to which doses of Fraction III equivalent to 0.2 gm. of yeast were given each day, and Rats 5039 and 5131, to which similar doses of Fraction IV were given, gained so little weight that it is evident that neither of these fractions contained as large a proportion of the water-soluble vitamine of the original yeast as did Fraction II. The slight gain of weight made by the rats having Frac-

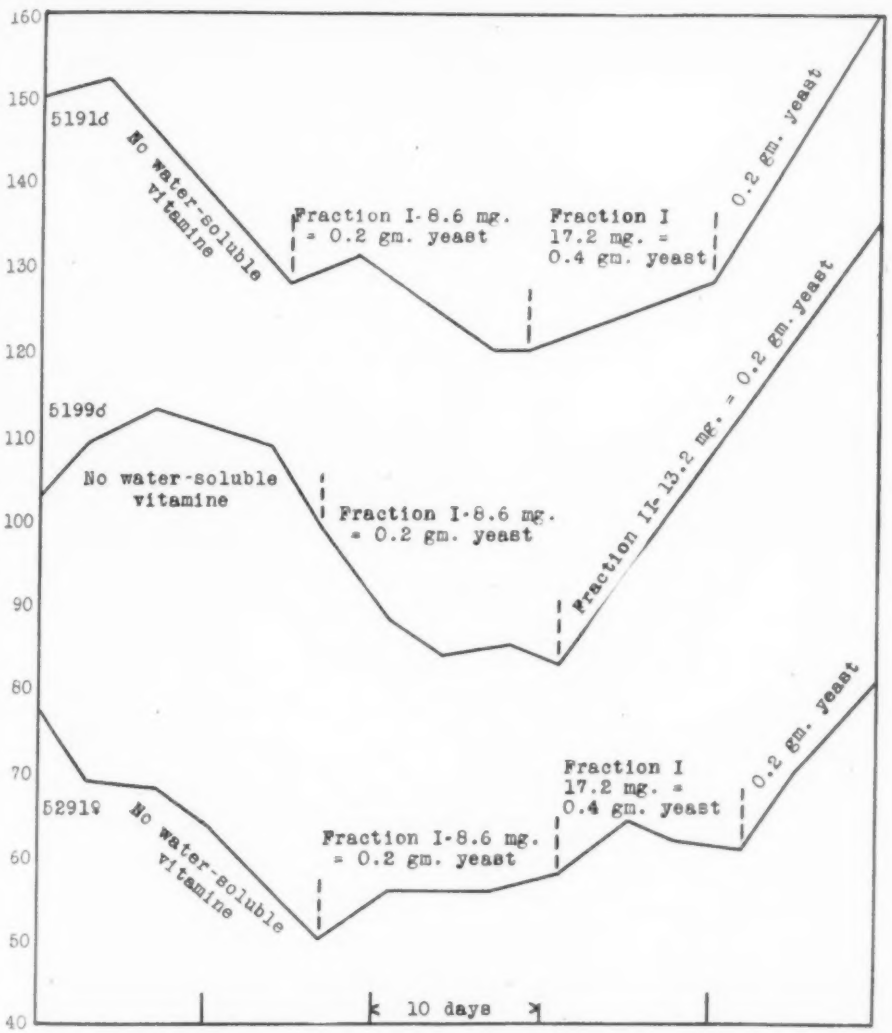

CHART II. Young rats after declining on a diet free from water-soluble vitamine fail to recover when quantities of Fraction I, equivalent to 0.2 gm. of yeast are added daily to their diets, but recover when given similar proportions of Fraction II or 0.2 gm. of yeast. Even doubling the quantity of Fraction I caused very little gain in weight.

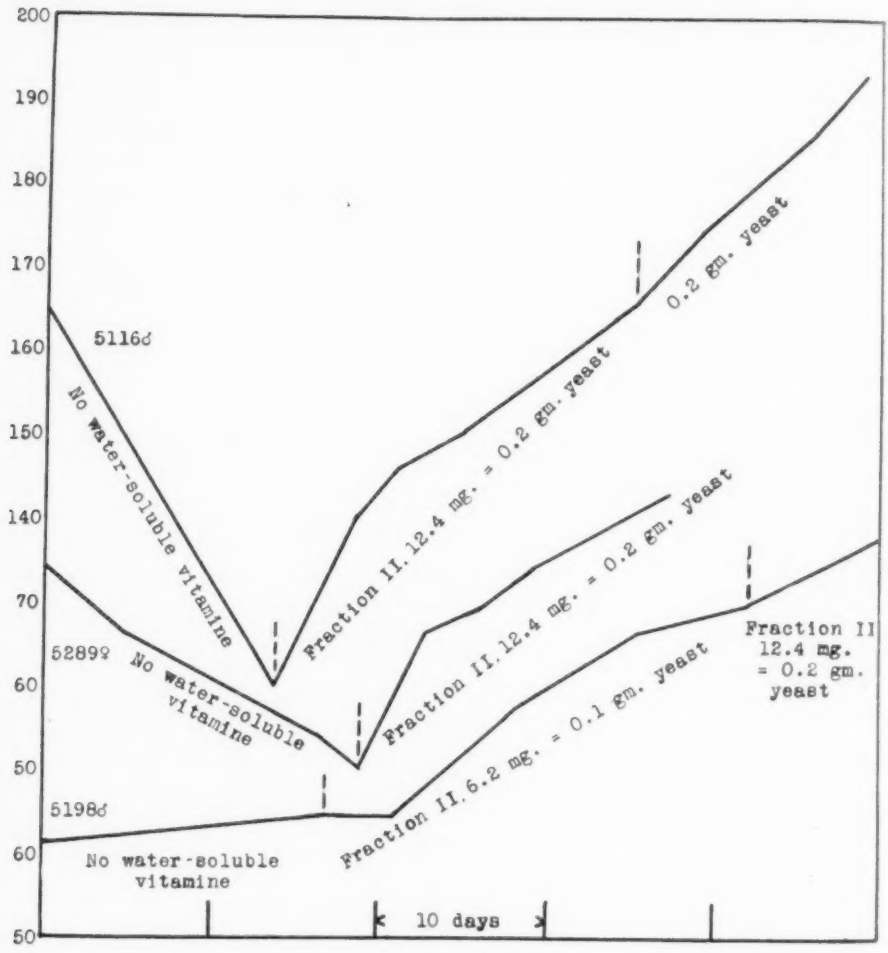

CHART III. Young rats declining on a diet free from water-soluble vitamine recover when given quantities of Fraction II equivalent to 0.2 or 0.1 gm. respectively of yeast. When 0.2 gm. of yeast was given to Rat 5116 instead of Fraction II it did not gain any faster.

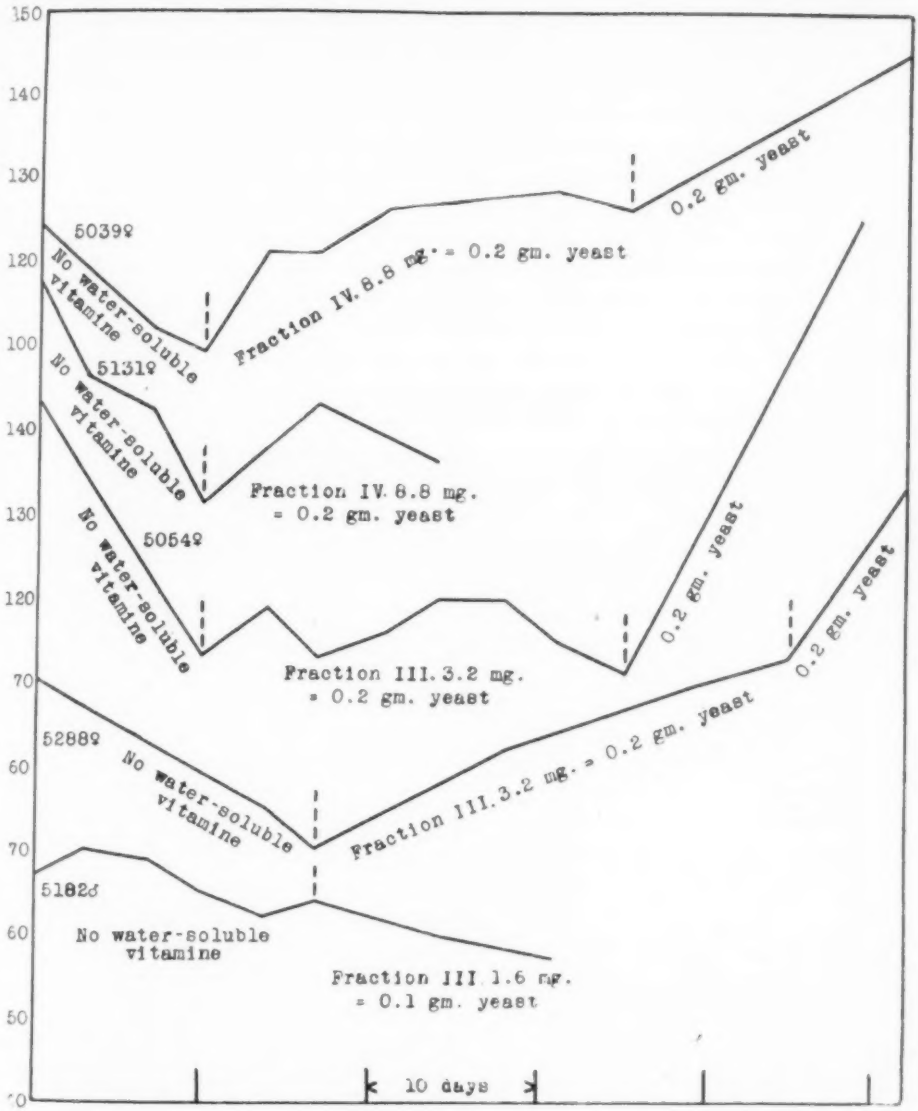

CHART IV. Young rats declining on a diet free from the water-soluble vitamine fail to recover when given quantities of Fractions III or IV equivalent to 0.2 gm. of the original yeast. Recovery is prompt when 0.2 gm. of yeast is given.

tions III and IV indicates, however, that these fractions contained some of the water-soluble vitamine. In view of the fact that daily doses of only 3.2 mg. of Fraction III enabled Rat 5288 to make a relatively considerable gain of weight shows that this fraction contained, weight for weight, as much, if not more, of the water-soluble vitamine as did Fraction II. However, since Fraction III amounted to only 1.6 per cent of the yeast it contained only a relatively small part of the total water-soluble vitamine. There can be no question therefore that Fraction II contained most of the water-soluble vitamine originally present in the yeast.

Having thus found that the greater part of the water-soluble vitamine can be concentrated in about 6 per cent of the yeast solids it is now possible to supply enough of this vitamine for normal nutrition without introducing such relatively large quantities of nitrogenous substances of unknown nature as have heretofore usually been necessary. Whether the method of fractionation here described is superior to precipitation by adsorption on Lloyd's[1] reagent remains to be determined, but it would seem as if our Fraction II offered advantages for the further study of many problems concerning the water-soluble vitamine. According to Seidell, Lloyd's reagent adsorbs 4.5 per cent of the nitrogen from the autolyzed yeast filtrate simultaneously with the vitamine, whereas our Fraction II contained 4.46 per cent of the total yeast nitrogen or 31.8 per cent of the nitrogen of the yeast extract.

As this Fraction II is easily prepared in large quantities it will certainly afford a better crude material for further study than does the autolyzed yeast filtrate which contains a large proportion of the products of autolysis derived from the yeast protein which, as our experiments show, are not concerned in the activity of vitamine preparations made from yeast. The chief advantage of the procedure here described lies in the preparation of the aqueous extract, for by avoiding autolysis the proportion of water-soluble constituents of the extract is reduced to a minimum while the quantity of water-soluble vitamine in the extract is apparently not diminished.

[1] Seidell, A., *J. Biol. Chem.*, 1917, xxix, 145.

It will be a matter of interest to learn more than is now known about the chemical nature of the constituents of this water extract of the living yeast, not only in connection with further attempts to concentrate the water-soluble vitamine, but also as a contribution to the chemistry of cytoplasm.

As yet we have made only a preliminary examination of this interesting mixture. That it consists chiefly of nitrogenous substances is indicated by its content of about 8.5 per cent of nitrogen. Proteins are not present in amounts detectable by saturating with ammonium sulfate, or by potassium ferrocyanide and acetic acid. A biuret reaction has been obtained only by very carefully applying this test to the part precipitated by 52 per cent alcohol. Inorganic constituents are also abundant, because the ash forms over 20 per cent of the dry solids. The different types of nitrogen in the water extract in percentage of the total nitrogen are shown in Table II.

TABLE II.

Types of Nitrogen in Per Cent of Total Nitrogen.

	Humus N.	NH_3 N.	NH_2 N.	Basic N.	Purine N.
Before hydrolysis.....	0	0	42	50	0
After "	0.64	1.56	60	50	18

The above figures indicate the presence of relatively large proportions of nucleic acid, amino-acids, and peptides, but as yet none of these has been isolated. Phosphatides are probably absent, because the ether extracts from Fractions II, III, and IV, the latter soluble in strong alcohol, failed to give any precipitate when poured into acetone.

Fraction II which contains most of the water-soluble vitamine presents greater interest than the aqueous extract. This fraction is very soluble in water, its solution being distinctly acid to litmus. Relatively considerable quantities of alkali are needed to produce a neutral reaction to litmus and not a little more must be added before an alkaline reaction results. Only a trace of a precipitate separates from the neutralized solution.

The unneutralized solution gives a large precipitate with lead acetate. Barium chloride causes only a turbidity, but yields an

abundant precipitate when the solution is previously neutralized with sodium hydroxide. Silver nitrate does likewise.

Baryta solution gives a voluminous precipitate which contains about 25 per cent of the solids of the fraction and a relatively small part of its nitrogen. About 25 per cent more of the fraction is thrown out of the alkaline filtrate from the barium precipitate by silver nitrate. This precipitate, when thoroughly washed with baryta solution, contains nearly one-half the nitrogen of the fraction. The aqueous solution of Fraction II when acidified with sulfuric acid yields a very large precipitate with phosphotungstic acid. Mercuric chloride gives a precipitate; copper sulfate gives none. An aqueous or alcoholic solution of picric acid gives a precipitate if enough is added. Whether any one of the above precipitates contains some or all of the water-soluble vitamine remains to be determined.

Such preliminary observations indicate that a variety of substances are present in this fraction, but give no clue to the nature of the water-soluble vitamine. We have provided ourselves with several kilos of this active yeast fraction and are now engaged in a systematic study of its constituents.

CRYSTALLINE SALTS OF URIDINPHOSPHORIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, October 31, 1919.)

Uridinphosphoric acid was previously described in form of its crystalline brucine and barium salts.[1] The identification of a nucleotide in form of its brucine salts was found not sufficiently reliable. The crystallization of the barium salt of uridinphosphoric is a slow and tedious process. Hence it was desirable to find such salts of the nucleotide which could be crystallized with readiness when one is in possession even of only a small quantity of material. In this communication are described several such salts; namely, the mono- and the diammonium salts of uridinphosphoric acid, the neutral lead salt, and the brucine salt prepared from the crystalline ammonium salt.

The neutral ammonium salt is readily prepared from the brucine salt and crystallizes as heavy, elongated prisms. In aqueous solution its optical rotation is $[\alpha]_D^{20} = +21.0$. It crystallizes with one crystal water.

The mono-basic salt crystallizes in form of long, prismatic needles. It is very soluble in cold and hot water, and in hot glacial acetic acid. In aqueous solution it has an optical rotation of $[\alpha]_D^{20} = +13.0$. The air-dry substance contains no crystal water.

The neutral lead salt crystallizes in long needles, and was found very useful in obtaining pure uridinphosphoric acid when the original brucine salt had not been sufficiently purified.

The brucine salt was prepared in order to establish the constants of the compounds, since there was no conclusive evidence as to the absolute purity of the brucine salt obtained on fractionation of the mixed brucine nucleotides derived from the products of hydrolysis of nucleic acid.

[1] Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 21; *J. Biol. Chem.*, 1918, xxxiii, 229.

EXPERIMENTAL.

Di-basic ammonium salt was prepared by decomposing with aqueous ammonium hydroxide a solution of the brucine salt of uridinphosphoric acid in 35 per cent alcohol. The brucine salt of the mononucleotide was obtained either on acid or on ammonia hydrolysis of yeast nucleic acid.

The crude ammonium salt was dissolved in a minimum volume of hot water and to the solution hot methyl alcohol was added to very slight opalescence. On standing over night a deposit of heavy crystals formed. In subsequent experiments crystallization began immediately after addition of alcohol, if the solution was seeded with a crystal of the pure substance. The substance decomposed at 185°C. (uncorrected).

0.0994 gm. of the substance gave 0.1048 gm. of CO_2 and 0.0486 gm. of H_2O.

0.1000 gm. of the substance analyzed for Kjeldahl nitrogen estimation required 10.68 cc. of 0.1 N acid.

0.3000 gm. of the substance gave 0.0870 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{13}N_4PO_9 + \frac{1}{2} H_2O$. *per cent*	Found. *per cent*
C	29.11	28.75
H	5.42	5.47
N	15.22	14.95
P	8.44	8.08

The optical rotation of the substance was as follows.

$$[\alpha]_D^{20} = \frac{+ 0.42 \times 100}{1 \times 2} = + 21.0$$

Mono-basic ammonium salt was prepared in the following way: 2.0 gm. of the neutral salt were dissolved in 15.0 cc. of glacial acetic acid, and to the hot solution hot ethyl acetate was added dropwise. Care was taken to wait with further addition until the precipitate forming on contact of ethyl acetate with the solution had disappeared. After a slight opalescence was established, the solution was allowed to stand over night. A crystalline sediment was found in form of balls consisting of long needles. In subsequent experiments, if the solution in glacial acetic was seeded with the pure substance, crystallization began immedi-

ately. The air-dry substance contracted at 210°C. (corrected) and decomposed at 242°C. (corrected).

0.1000 gm. of the substance gave 0.1142 gm. of CO_2 and 0.0452 gm. of H_2O.

0.1000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 8.8 cc. of 0.1 N acid.

0.3000 gm. of the substance gave 0.0943 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{16}N_3PO_9$. *per cent*	Found. *per cent*
C	31.66	31.14
H	4.73	5.05
N	12.32	12.32
P	9.09	8.76

The optical rotation of the aqueous solution was as follows.

$$[\alpha]_D^{20} = \frac{+ 0.26 \times 100}{1 \times 2} = + 13.0$$

Neutral lead salt was prepared in the following way: 2.0 gm. of the neutral ammonium salt were dissolved in 50 cc. of water; to the solution 10 cc. of glacial acetic acid were added, and to the hot solution of the nucleotide a hot solution of neutral lead acetate was added. Immediately a gelatinous precipitate formed which, on boiling, disappeared nearly completely. The solution was filtered and seeded with a few crystals obtained from a test-tube experiment. Crystallization in long needles began immediately. The crystals once formed are very little soluble in boiling water.

Dried to constant weight the substance had the following composition.

0.1070 gm. of the substance gave 0.0788 gm. of CO_2 and 0.0206 gm. of H_2O.

0.1856 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 6.90 cc. of 0.1 N acid.

0.2782 gm. of the substance gave 0.0573 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{11}N_2PO_9Pb$. *per cent*	Found. *per cent*
C	20.40	20.08
H	2.10	2.21
N	5.29	5.21
P	5.86	5.74

Brucine Salt of Uridinphosphoric Acid.—About 4.0 gm. of the neutral ammonium salt were converted into the lead salt. This was freed from lead by means of hydrogen sulfide and the brucine salt was obtained in the usual way. The salt was recrystallized twice, each time out of 1,500 cc. of 35 per cent alcohol.

The air-dry substance effervesced without becoming transparent at 185°C. (corrected) and contracted and melted without further decomposition at 195°C. (corrected).

The air-dry substance had the following composition.

0.0996 gm. of the substance gave 0.1948 gm. of CO_2 and 0.0591 gm. of H_2O.

0.2000 gm. of the substance gave 12.8 cc. of nitrogen gas at $T° = 24°C.$, at $P = 759$ mm.

0.3000 gm. of the substance gave 0.0270 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{13}N_2PO_9(C_{22}H_{26}N_7O_{14})_2+7H_2O$. *per cent*	Found. *per cent*
C	53.30	53.33
H	6.43	6.64
N	6.79	7.35
P	2.50	2.51

The rotation of the substance, owing to the insolubility of the substance, was taken in great dilution, and was as follows.

$$[\alpha]_D^{20} = \frac{-0.16 \times 100}{2 \times 0.4} = -20.0$$

ON THE IDENTITY OF THE WATER-SOLUBLE GROWTH-PROMOTING VITAMINE AND THE ANTINEURITIC VITAMINE.

By H. H. MITCHELL.

(*From the Department of Animal Husbandry, University of Illinois, Urbana.*)

(Received for publication, October 13, 1919.)

The vitamine requirements of animals have been investigated along two distinct lines; *i.e.*,(1) through a study of the nutritive deficiencies of rations containing only known chemical compounds, and (2) through a study of the prevention and cure of certain diseases definitely referable to faulty diet. By the first line of investigation it has been repeatedly demonstrated that, besides the well known constituents of plant and animal tissues, the higher mammals require for the maintenance of health, and even of life, and for the production of normal growth small amounts of substances of unknown composition, more or less widely distributed throughout natural food products. At least two such substances are required. One is associated with certain animal fats and fresh leafy vegetables especially, and variously referred to as the fat-soluble vitamine or growth-promoting substance, or as fat-soluble A, a convenient abbreviated term introduced by McCollum and Kennedy, which unfortunately has not the general currency that it deserves. The other growth-promoting substance is never associated with fats, but is especially rich in the glandular tissues of animals, hens' eggs, the embryos of seeds, and the leaves of plants. It is spoken of as the water-soluble growth-promoting vitamine or as water-soluble B (McCollum). Any assertion that the common sources of either of these vitamines contain only one indispensable substance is based purely upon circumstantial evidence.

By the second line of investigation it has been shown with reasonable certainty that there are at least two diseases referable to faulty diet, the cause of which, in each case, is a deficiency in

the diet of an unknown substance associated with natural food products. These two diseases, beri-beri and scurvy in man (or polyneuritis in pigeons and scurvy in guinea pigs), are "deficiency diseases" in the sense that they may result when an animal subsists on food containing all the known and well recognized constituents of a complete diet, and may be cured, except for serious organic lesions, by adding to such a diet small amounts of extracts of certain natural food products. The unknown substances involved in the production of these two deficiency diseases have been called the antineuritic vitamine and the antiscorbutic vitamine, respectively, and their distribution in plant and animal tissues, their solubilities, and chemical properties have been the subject of a large amount of research. Besides these two deficiency diseases, no others are generally recognized as such. A condition of sore, inflamed eyes, which, according to McCollum, should be diagnosed as xerophthalmia, often results from a deficiency of sources of fat-soluble A in the diet. While McCollum definitely classifies this condition as a deficiency disease, it does not seem to be generally recognized as such, and a reasonable doubt may exist as to whether it invariably results from a deficiency of fat-soluble A and whether its etiology does not involve an infection avoidable by exercising proper sanitary measures alone.[1]

It is evident that the numerous recent investigations on vitamines that have been undertaken by many workers, especially in America and England, have seriously complicated the problem of the nutritive requirements of animals. Any experimental evidence that would simplify this situation would therefore be doubly welcome. However, an undue simplification, based upon insufficient evidence, would lead to confusion and might seriously impede progress. One possibility that would simplify matters would be a clear-cut demonstration that two or more of these vitamines are in fact identical. That fat-soluble A and water-soluble B are identical, or that the antineuritic and antiscorbutic vitamines are identical, may be ruled out at once on the evidence at present available. However, the identity of water-soluble B and the antineuritic vitamine is a possibility not definitely dis-

[1] Bulley, E. C., *Biochem. J.*, 1919, xiii, 103.

posed of in this summary manner. In fact, the belief exists in many quarters that such an identity exists. This belief ranges from positive conviction, through tacit acceptance, to a frank weighing of probabilities. On the one hand is the statement of McCollum and his coworkers that: "Xerophthalmia and polyneuritis are abundantly demonstrated to have their origin in the lack of a sufficient amount of the fat-soluble A and water-soluble B respectively in the diet."[2] Evidently as the result of this unequivocal attitude of McCollum, some investigators use the terms "water-soluble B" and "antineuritic vitamine" interchangeably. Osborne and Mendel are more conservative: "Whether or not the antineuritic component [of yeast] is identical with the growth-promoting one is a question which as yet has received no definite answer. . . . ."[3] Again, after referring to studies of the protective, curative, or antineuritic properties of certain animal tissues, they say: "That the substance which induces the remarkable recoveries which have been described in these cases is identical with the water-soluble hormone which is so essential for growth and maintenance is as yet merely a matter of conjecture."[4]

In view of the importance of the question of the identity of these two vitamines, one essential for the maintenance of life and growth, the other for the prevention of multiple neuritis, and in view of the lack of general acceptance of the affirmative statement of McCollum and coworkers, a somewhat searching critical consideration of the evidence may render a real service in clarifying the issue and indicating the most likely points of future attack.

The conclusion that the two vitamines are identical seems to be based upon the following grounds. (1) The distribution of the two substances in natural food products is very similar and the correlation between the actual amounts (in as far as these have been measured by biological tests) found in different products appears to be close. (2) The lack of known sources of water-soluble B in the diet of various species of experimental animals

[2] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1918, xxxiii, 413.

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 154.

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 311.

seems generally, if not always, to result in symptoms of nerve degeneration and paralysis. (3) Extracts of natural food products possessing growth-promoting properties are said to contain very probably only one indispensable vitamine, though supplementing satisfactorily a ration containing no other possible source of antineuritic vitamine. (4) The solubilities of the two vitamines in the common solvents are said to be identical. (5) Attempts at isolating the two vitamines have shown that they possess identical precipitants and adsorbents. (6) The stabilities of the two substances, especially to acids, alkalies, and elevated temperatures, seem to be similar if not identical.

Critical analyses of these points are presented in order.

1. While the distribution of the two vitamines among natural food products is strikingly similar in many respects, there are some instances where the correlation is not close. Through the work of Osborne and Mendel,[5] it has been shown that green vegetables, such as cabbage and spinach, are rather rich sources of water-soluble B, compared, for example, with the whole cereal grains. McCollum and Kennedy,[6] however, have found that cabbage contains the antineuritic factor in "a quite low concentration," a conclusion confirmed by some recent work of Chick and Hume.[7] These investigators have concluded from experiments on the relative vitamine content of a large range of different foodstuffs that fresh and desiccated vegetables, including the cabbage, onion, and carrot, are poor sources of the antineuritic vitamine as compared with whole wheat, being about on a par with fresh meat. Fresh meat is known to be a poor source of both water-soluble B and the antineuritic vitamine,[8] but, on the other hand, the carrot seems to be rich in water-soluble B,[9] though poor in antineuritic vitamine. Again, Chick and Hume have found the potato to have a very low content of antineuritic vitamine, having practically no value as a preventive against avian

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187.

[6] McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 496.

[7] Chick, H., and Hume, E. M., *Tr. Soc. Trop. Med. and Hyg.*, 1916–17, x, 156.

[8] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309. Cooper, E. A., *J. Hyg.*, 1912, xii, 436; 1914, xiv, 12.

[9] Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxvi, 171, 191.

polyneuritis, a result confirmed by Vedder and Clark,[10] though McCollum and Kennedy claim that potato juice possesses a moderate curative power. As regards its content of water-soluble B, however, McCollum, Simmonds, and Parsons[11] have shown that the potato, when constituting 84.5 per cent of the ration of rats, provides enough of this vitamine for normal growth. Chamberlain, Vedder, and Williams[12] found water extract of onions to have no curative effect on polyneuritic pigeons, though Osborne and Mendel[13] discovered considerable water-soluble B in this food as well as in turnips, beets, and tomatoes.

The green vegetables, and roots and tubers seem in general, therefore, to contain rich or moderate amounts of the growth-promoting water-soluble vitamine, and small amounts or none at all of the antineuritic vitamine. If this relation is abundantly confirmed by future research, it may of itself effectively dispose of any contention of the identity of the two vitamines. The evidence as it stands, however, does not amount to finality for several reasons. Much recent work has shown that the vitamine content of fresh vegetables, and roots and tubers is extremely variable, depending apparently on the freshness and maturity of the material. No experiments appear to have been carried out on the antineuritic and the growth-promoting properties of the same vegetable samples. Again, the experimental polyneuritis of pigeons is almost invariably induced by a diet of polished rice in investigations on the comparative antineuritic properties of foods. Polished rice, however, is defective in several factors besides the antineuritic factor. It is therefore probable that foods containing comparable amounts of this vitamine may be unequally effective in preventing or curing the polyneuritic symptoms, depending upon the extent to which they supplement polished rice in these other respects. Conversely, foods containing unequal concentrations of the antineuritic principle may have their relative values as sources of the vitamine distorted for

[10] Vedder, E. B., and Clark, E., *Philippine J. Sc., Section B*, 1912, vii, 423.

[11] McCollum, E. V., Simmonds, N., and Parsons, H. T., *J. Biol. Chem.*, 1918, xxxvi, 208.

[12] Chamberlain, W. P., Vedder, E. B., and Williams, R. R., *Philippine J. Sc., Section B*, 1912, vii, 45.

[13] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxix, 29.

the same reason. The argument is illustrated by the experience of Campbell and Chick with scorbutic rations.[14] A ration of oats, bran, and water produces death from scurvy in guinea pigs in 3 to 4 weeks. However, the addition of 60 cc. of autoclaved milk, a food containing no demonstrable antiscorbutic value, delays the onset of scurvy from 1 to 3 weeks, evidently simply by correcting the deficiencies of the basal ration in factors other than the antiscorbutic.

Further evidence, out of harmony with the assumption that water-soluble B and the antineuritic vitamine are identical, is afforded by experiments on unpolished rice. McCollum and Davis[15] have shown reason to believe that unhusked rice is adequately supplemented by casein, salts, and butter fat, similar to the other cereal seeds. Gibson and Concepcion,[16] however, report experiments on pigeons indicating an incomplete protection against polyneuritis afforded by an exclusive diet of unhusked rice. Six fowls were fed on palay (unhusked rice), three for 2 months, two for 3 months, and one for 4 months. While they developed no symptoms of neuritis in this time, on postmortem examination, the sciatic nerves showed distinct degenerative changes in every case on being stained by the Marchi method. The degeneration corresponded to that obtained with birds fed for 2 weeks or more on milled rice. In fact, the degeneration in two of the birds was more pronounced than can be observed in some subjects that have died of rice polyneuritis. In the case of man, also, analogous results have been reported.[17]

2. The lack of known sources of water-soluble B in the diet of rats has frequently been said to result in symptoms of paralysis of the hind legs, and the conclusion has been drawn that a well defined neuritis existed. Such symptoms, however, are not universally noted as a result of subsistence on rations totally lacking in water-soluble B,[18] and the conclusion that they invariably

[14] Campbell, M. E. D., and Chick, H., *Lancet*, 1919, ii, 320.

[15] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 230.

[16] Gibson, R. B., and Concepcion, I., *Philippine J. Sc., Section B*, 1914, ix, 119.

[17] Strong, R. P., and Crowell, B. C., *Philippine J. Sc., Section B*, 1912, vii, 414. Shibayama, *J. Trop. Med. and Hyg.*, 1913, xvi, 284.

[18] Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

develop cannot be verified. Even when animals do develop such symptoms it does not seem to be specific to a deficiency of this vitamine. Hart, Miller, and McCollum[19] report experiments on pigs on a large number of rations, leading in many cases to paralysis of the hind quarters, and, as brought out by the histological examination of sections of the spinal cord, to marked edema and degeneration of the motor cells. They conclude from this work:

"Malnutrition, histologically characterized by nerve degeneration, may result from the absence of certain factors in the diet as in the case of beri-beri. A similar condition may likewise arise from the presence of toxic materials in apparently normal food products, and in the presence of all known factors essential for continued growth and well-being."

It can hardly be contended, therefore, that the appearance of neuritic conditions in experimental animals is an unequivocal indication of a deficiency in antineuritic vitamine.

3. The existence of two indispensable unknown substances in water and alcohol extracts of natural foods is doubted by McCollum and Simmonds[20] on the basis of the following evidence.

"In the experimental part of this paper it is shown that the water-soluble B is not extracted directly from beans, wheat germ, or pig kidney by ether, benzene, or acetone, but is readily extracted in great part by alcohol. After being removed by alcohol it is shown to be soluble in benzene, but very slightly soluble in acetone. The probability that there should be two or more physiologically indispensable substances in what we term water-soluble B, both or all of which should show the same solubility relations with three solvents, is relatively small and lends support to our view that the substance which protects animals against polyneuritis is the only essential complex in the extracts described."

While the argument is directed particularly against the theory that scurvy is a deficiency disease, it is just as forceful against any theory that water-soluble B and the antineuritic vitamine are not one and the same thing. Thus, McCollum's theory of the nutritive requirements of animals, which, because of its simplicity and the unequivocal terms in which it is stated, has

[19] Hart, E. B., Miller, W. S., and McCollum, E. V., *J. Biol. Chem.*, 1916, xxv, 239.

[20] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 62.

gained many advocates, has no room for two vitamines possessing the solubilities of water-soluble B. The argument is not particularly impressive, being entirely of a circumstantial character. In fact, it is quite conceivable that the treatment of natural foods with boiling alcohol breaks up combinations of vitamines with other substances, so that removal of the water-soluble B by this solvent may have been a combination of chemical and purely solvent action. A crucial experiment that would dispose of this possibility would have been to boil the food with alcohol in a reflux condenser for a short time only, evaporate off the alcohol, and then test with acetone and benzene for extraction of the active substance.

4. The solubilites of the water-soluble B and the antineuritic vitamine are very largely similar. Both are soluble in water and dilute alcohol and are ordinarily stated to be insoluble in fat solvents. Solubility in absolute alcohol is variously stated for both substances. Osborne and Mendel, and Drummond[21] claim that the water-soluble growth-promoting substance of yeast is insoluble in absolute alcohol, and McCollum and Simmonds[20] have found it to be only incompletely soluble in 95 per cent alcohol. On the other hand, Eijkman[22] claims that the antineuritic substance of yeast is extracted by strong alcohol. The neuritic curative substance of rice polishings has been repeatedly extracted by Funk[23] and Fraser and Stanton[24] by absolute alcohol and appears to be readily soluble in this reagent. Cooper[8] has also shown that the antineuritic vitamine of dried beef is extracted by absolute alcohol, and that the constituent of egg yolk that cures polyneuritis in pigeons is readily extractable with ether[25] from the dried yolk. After extraction with ether, a further yield was obtained from the residue by means of absolute alcohol.

While the evidence for the solubility of the two vitamines in alcohol is incomplete and somewhat conflicting, though lending

[21] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 158. Drummond, J. C., *Biochem. J.*, 1917, xi, 261.

[22] Eijkman, C., *Arch. Schiffs- u. Tropenhyg.*, 1911, xv, 698.

[23] Funk, C., *J. Physiol.*, 1911–12, xliii, 395.

[24] Fraser, H., and Stanton, A. T., *Lancet*, 1910, ii, 1755.

[25] McCollum and his coworkers have repeatedly shown that water-soluble B is not extracted by ether from food materials.

support to the conclusion that the growth-promoting factor is considerably less soluble than the antineuritic, some clear-cut evidence exists of a distinct difference in solubility in acetone and benzene, mainly from the work of McCollum and his associates. Thus, McCollum and Kennedy[26] conclude from many experiments on the curative properties of various extracts of wheat embryo for polyneuritic pigeons that acetone and benzene extract from this material, previously rendered fat-free by extraction with ether, the substance which relieves the symptoms of polyneuritis in pigeons. Alcohol (95 per cent), water, acetone, and benzene extracts of fat-free wheat embryo were all shown to be capable of curing polyneuritic pigeons, and, as far as can be judged from the protocols of the experiments, the cure was just about as readily accomplished with acetone and benzene extracts as with alcohol and water extracts. At most, the superiority of the latter was slight.

Entirely different results were obtained by McCollum and Simmonds[27] in later investigations on the solubility of water-soluble B. In investigating the growth-promoting properties of extracts of raw and cooked navy beans, wheat embryo, and pig kidney added to a basal ration deficient only in water-soluble B, they were able to show that acetone and benzene do not extract the water-soluble B from these products to any appreciable extent. Slight indications that the acetone extract carried traces of this vitamine were encountered, but they were still led to believe that most of the substance remained in the residue. This work confirmed some previous work by McCollum and Davis[28] on the supplementary action of acetone extracts of wheat embryo on polished rice. After extraction of the water-soluble B by alcohol, it was found by McCollum and Simmonds to be only slightly soluble in acetone, and many times more soluble in benzene.

[26] McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

[27] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

[28] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 229, 230. This investigation and the preceding one are open to the criticism that no food intake records are given in order that the reader may judge whether a change in ration was effective by reason of a difference in food intake or of a difference in food composition, and also that the experimental periods were often too short to indicate any effect at all.

Recently Steenbock[29] has reported that by means of neutral solvents there was prepared a water-acetone-soluble fraction from egg yolk which in small doses by intraperitoneal injections was able to cure a pigeon suffering from polyneuritis.

From the work quoted, therefore, the evidence lends support to the conclusion that the water-soluble B is not extracted from food materials to any appreciable extent by either acetone or benzene, and that when extracted it is still only slightly soluble in acetone, though readily soluble in benzene. The antineuritic vitamine, however, seems to be readily extracted from wheat embryo by acetone and benzene, and to be readily soluble in both of these solvents.

5. Attempts to isolate the antineuritic vitamine from rice polishings and yeast have been much more numerous and have given more definite results than similar attempts with the water-soluble B. The antineuritic vitamine has been shown to be quantitatively precipitated by phosphotungstic acid in 5 per cent sulfuric acid solution, giving a phosphotungstate insoluble in acetone. It is also completely precipitated by silver nitrate and barium hydroxide, only partially precipitated by mercuric chloride, and is not precipitated by platinic chloride. It is quantitatively adsorbed by animal charcoal, fullers' earth, and by hydrated aluminium silicate (Lloyd's reagent). The antineuritic vitamine is also dialyzable.

Funk and Macallum[30] have attempted a similar fractionation of the water-soluble B from yeast with indifferent success. They showed that phosphotungstic acid precipitated the growth-promoting substance, and that the filtrate from this precipitation was inactive. However, only a small fraction of the growth-promoting capacity of the yeast was found to have survived this precipitation. Subsequent decomposition of the precipitate and reprecipitation with silver nitrate and baryta precipitated a fraction, whose growth-promoting effect was "not sufficiently marked to encourage further investigation." Lloyd's reagent was also used as a precipitant without much success, as the rats on the filtrate also showed increments in growth. According to the authors:

[29] Steenbock, H., *J. Biol. Chem.*, 1917, xxix, p. xxvii.

[30] Funk, C., and Macallum, A. B., *J. Biol. Chem.*, 1916, xxvii, 63.

"The results obtained so far clearly indicate that the growth-promoting substance is analogous to and possibly identical with the beri-beri vitamine. . . . . However, it must be admitted that while it is uncertain whether these two substances are chemically different, the results obtained do not exclude such a possibility."

Eddy[31] showed that the water-soluble portion of the alcoholic extract of sheep pancreas contains a substance capable of inducing marked increase in growth when added to a ration very low, if not lacking, in vitamines. This substance was removed from the extract with Lloyd's reagent, though the completeness of removal was not tested. It was also precipitated by phosphotungstic acid. A criticism of the work of Funk and Macallum and of Eddy is that the basal ration used in making the biological tests, besides their deficiency of water-soluble B, could not have contained more than a trace of fat-soluble A.

A more extensive and better planned experiment was reported by Drummond,[32] investigating the water-soluble B from yeast. Drummond was able to show that the growth-promoting substance is dialyzable. From the dialysate phosphotungstic acid precipitated a fraction possessing very slight growth-promoting activity, while the unprecipitated fraction possessed none. The distinction between the two fractions was slight and in the absence of food intake records is not susceptible of unequivocal interpretation. In another experiment, better growth curves were obtained when the fraction represented by the phosphotungstates insoluble in acetone was used than when that contained in the phosphotungstates soluble in acetone was used. Even smaller differences were observed between the growth curves of these two groups, however, than in the preceding experiment. Upon fractionation of yeast dialysate with silver nitrate and baryta, the precipitate produced with silver nitrate (purine fraction) had no effect on growth, while that produced on the further addition of baryta (pyrimidine fraction) contained small traces of vitamine.

While Drummond's results support the view that water-soluble B and the antineuritic vitamine are identical, they do not constitute a demonstration. The lack of food intake records complicates their interpretation, while the large losses of vita-

[31] Eddy, W. H., *J. Biol. Chem.*, 1916, xxvii, 113.
[32] Drummond, J. C., *Biochem. J.*, 1917, xi, 255.

mine as soon as precipitation was resorted to finds no parallel in analogous work with the antineuritic substance. For example, Funk showed that the water solution of an alcoholic extract of rice polishings when tested on pigeons suffering from polyneuritis was effective in doses corresponding to about 20 gm. of the original polishings. On precipitation of the solution with phosphotungstic acid, decomposition of the precipitate with baryta, precipitation of the excess barium with sulfuric acid, neutralization and evaporation *in vacuo*, and extraction of the residue with absolute alcohol, the extract was found to be effective as a curative for polyneuritic pigeons in doses corresponding to about 40 gm. of the original polishings. This would indicate a loss of some 50 per cent in antineuritic efficiency, a much smaller loss than seems to result with water-soluble B, even when the manipulations after the phosphotungstic acid precipitation are simplified by decomposing the precipitate with an amyl-alcohol-ether mixture, and eliminating the absolute alcohol extraction. The ready adsorption of water-soluble B by precipitates of all descriptions postulated by Drummond, in explaining the large losses during chemical manipulation, does not seem to be a property of the antineuritic vitamine. Thus, Emmett and McKim[33] show that while this vitamine is adsorbed by fullers' earth and Lloyd's reagent it is not adsorbed by the kieselguhrs or infusorial earths, indicating a selective adsorption by the former. Being readily dialyzable, there seems to be no compelling reason for believing it to be indiscriminately adsorbed by precipitates of all kinds.

6. The stability of both water-soluble B and the antineuritic vitamine to acids seems to be great. Even boiling the vitamines with concentrated mineral acids does not seem to destroy them to any appreciable extent, though some results reported by Drummond[32] may be interpreted as indicating a partial destruction of water-soluble B by boiling with 20 per cent sulfuric acid for 10 hours, a result not in harmony with the work of Funk on antineuritic vitamine. Both vitamines seem to be stable to even concentrated alkalies at room temperature.[34] At the boiling tempera-

[33] Emmett, A. D., and McKim, L. H., *J. Biol. Chem.*, 1917, xxxii, 409.

[34] The conclusion of Fraser and Stanton (Fraser, H., and Stanton, A. T., *Lancet*, 1915, i, 1021), to the effect that exposure of antineuritic preparations to 0.5 per cent sodium hydroxide at room temperature very quickly destroyed their curative properties for polyneuritic pigeons, has not been confirmed by recent investigations.

ture the antineuritic vitamine seems to be very rapidly destroyed by alkalies,[29] though experiments on this point are too few to warrant drawing definite conclusions. Water-soluble B does not seem to be destroyed particularly rapidly by dilute alkalies at high temperatures. While Drummond[32] found that hot 5 per cent sodium hydroxide in 5 hours tends to destroy the growth-promoting properties of yeast preparations, Osborne, Wakeman, and Ferry[18] were unable to detect the slightest destruction of this vitamine in dry brewers' yeast after digesting for 21.5 hours with 0.1 N sodium hydroxide and subsequently heating on the water bath for 2 hours. The conflicting results of McCollum and Simmonds,[20] and of Daniels and McClurg[35] on alkali of greater strength cannot at present be evaluated. For the proper solution of the question, food intake records must be considered.

While both water-soluble B and antineuritic vitamine do not seem to be destroyed by long exposure to a temperature of 100°C. or even slightly higher, the evidence is conflicting for temperatures of 120°C. Early investigations on the antineuritic vitamine in general substantiate the conclusion that 1 to 2 hours exposure to this temperature either totally destroys the antineuritic efficiency or markedly lowers it. The recent systematic investigation of Chick and Hume[36] indicates that the curative properties of wheat embryo and yeast extract are rapidly destroyed at 120°C. Wheat embryo, heated at 118–124°C. for 2 hours, failed to effect a complete cure of polyneuritis in pigeons when given in doses four times as large as the effective dose of unheated embryo. Heated at 110–117°C. for 40 minutes, it required more than twice as much embryo. Experiments with yeast extract showed a slower destruction of the antineuritic substance, since when heated at 120°C. for 2 hours 10 cc. of extract seemed to be as effective in curative action as 4 cc. of the unheated extract.

Unfortunately no strictly quantitative work on the effect of heat on water-soluble B, comparable to the investigations of Chick and Hume on the antineuritic vitamine, has been reported. McCollum and Davis[37] have shown that rats grow normally on

[35] Daniels, A. L., and McClurg, N. I., *J. Biol. Chem.*, 1919, xxxvii, 201.

[36] Chick, H., and Hume, E. M., *Proc. Roy. Soc. London, Series B*, 1917–19, xc, 60.

[37] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.

rations containing 10 per cent of evaporated whey, heated in an autoclave at 15 pounds pressure (120°C.) for 1 hour, as the sole source of water-soluble B. In view of the low content of milk in this vitamine it does not seem probable that any appreciable destruction could have occurred in this experiment. In another experiment, 13.3 per cent of wheat embryo heated in the autoclave for 1 hour served as the sole source of water-soluble B, supporting normal growth and reproduction. McCollum, Simmonds, and Pitz[38] showed that rations containing as low as 25 per cent of navy beans, moistened, and heated in the autoclave for 75 minutes at 15 pounds pressure, supported normal growth and reproduction in rats. Daniels and McC urg[35] heated navy beans, soy beans, and cabbage in a pressure cooker for 15 to 40 minutes at 120°C. and found them to be suitable sources of water-soluble B in the ration of rats when included in excessive amounts. No definite conclusions can be deduced from this experiment, however, as to the rate of destruction of the vitamine if any destruction occurred. Drummond[32] found that heating yeast dialysate for 30 minutes at 120°C. impaired its value as a source of water-soluble B, but the growth curves illustrating his experiment afford no basis for assuming any great destruction of vitamine, since only 6 per cent of the preparations tested was included in the rations as the sole source of water-soluble B.

The experiments just reviewed on the stabilities of the water-soluble growth-promoting vitamine and of the antineuritic vitamine, afford no sure basis of distinction though they are suggestive as indicating a greater stability of the former to hot alkali and to temperatures above 100°C.

In evaluating the data on the occurrence and properties of the two vitamines cited above, there seems to be very good reason for doubting their identity. In settling the question definitely, however, there is need of experiments in which different foods and preparations from foods are tested both for their growth-promoting properties and their curative effects on polyneuritic pigeons. Until such work is done in a quantitative way, dogmatic assertion that a lack of water-soluble B leads to polyneuritis or beri-beri, or an interchangeable use of the terms

[38] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 521.

"water-soluble B" and "antineuritic vitamine" serves no purpose, tending rather to impede progress in this direction. It is significant that while many investigators in nutrition regard the etiology of beri-beri as definitely cleared up, the medical profession in those parts of the world where beri-beri is endemic are not at all convinced of the fact, and still believe that a definite infection is involved along with malnutrition.[39] Furthermore, Gibson and Concepcion[16] say that in the Philippine Islands the therapeutic use of rice bran or extracts and preparations of rice bran has not given the specific results for man which were expected from theoretical considerations of the etiology of the disease, although the mortality has been greatly reduced. Williams and Johnston[40] also are veering away from the orthodox view of the cause of beri-beri in favor of a toxic view. They have been able to transmit polyneuritis to a pigeon, subsisting on unhusked rice, by feeding it the minced internal organs of birds that had died of the disease. This bird, 9 days after the ingestion of the diseased organs, developed all the typical symptoms of polyneuritis precisely as do birds fed on polished rice. The 2nd day thereafter the bird became completely prostrated, displayed increasingly severe retraction of the neck and labored breathing, and died. In view of such reports it cannot be doubted that many points in the etiology of polyneuritis and of beri-beri still need to be cleared up, and that premature deductions from a limited amount of experimental data do not hasten but can only defer the ultimate solution.

[39] See for example the discussion by medical men of the paper of Chick and Hume presented before the Society of Tropical Medicine and Hygiene (Chick, H., and Hume, E. M., *Tr. Soc. Trop. Med. and Hyg.*, 1916–17, x, 179–186).

[40] Williams, R. R., and Johnston, J. A., *Philippine J. Sc., Section B*, 1915, x, 337.

THE STRUCTURE OF YEAST NUCLEIC ACID.

IV. AMMONIA HYDROLYSIS.

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(Received for publication, November 1, 1919.)

The tetranucleotide theory of the structure of yeast nucleic acid was first enunciated by the writer[1] and was subsequently conclusively demonstrated by the experimental evidence furnished by Levene and Jacobs,[2] and by Levene and La Forge.[3] The facts on which the theory was based were: first, the isolation of four nucleosides; second, the isolation of simple pyrimidine nucleotides. These were obtained on partial hydrolysis of yeast nucleic acid. The third fact, important for the development of the theory, was the elucidation of the order of linkage of the components of one simple mononucleotide.[4]

The experimental data obtained until that phase of work permitted no rational formulation of the mode of linkage between individual mononucleotides. Our original graphic representation of the entire molecule of yeast nucleic acid had only an arbitrary schematic sense. In a publication on thymus nucleic acid, Levene and Jacobs made that point clear. Owing to pressure of other work, our own investigations into the problem of the linkage of the mononucleotides was making slow progress, when Thannhauser with his collaborators,[5] and Jones with his collaborators[6] entered the field of nucleic acid study.

[1] Levene, P. A., *Biochem. Z.*, 1909, xvii, 121.

[2] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1909, xlii, 2475, 2703; 1910, xliii, 3151; 1911, xliv, 1027.

[3] Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, xlv, 608, 3164.

[4] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1908, xli, 2704; 1909, xlii, 335, 1198.

[5] Thannhauser, S. J., *Z. physiol. Chem.*, 1914, xci, 329. Thannhauser, S. J., and Dorfmüller, G., *ibid.*, 1917, c, 121.

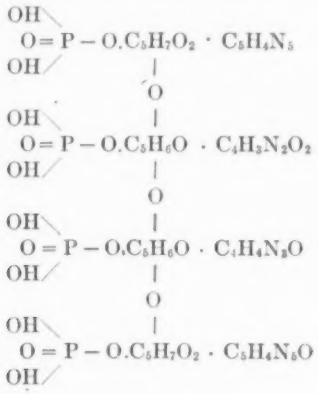
[6] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71. Jones, W., and Germann, H. C., *ibid.*, 1916, xxv, 93. Jones, W., and Read, B. E., *ibid.*, 1917, xxix, 123; xxxi, 39.

By means of either enzyme action or by methods of chemical hydrolysis, they obtained intermediate substances which were regarded by them as di- and trinucleotides.

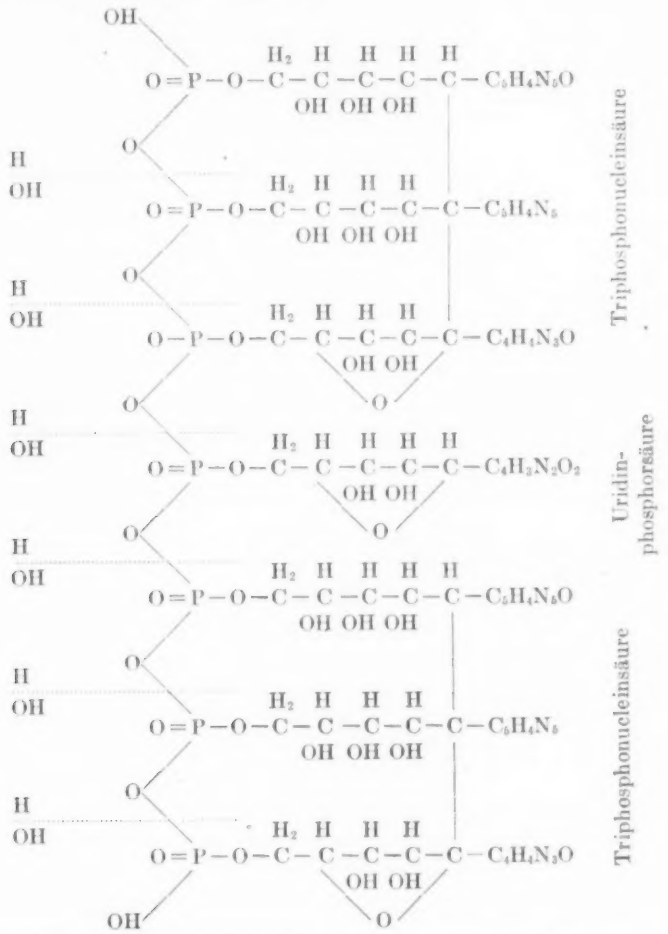
The methods of hydrolysis employed by them in some instances were identical with those employed by us in other instances, slightly modified. The method of separating simple nucleotides as their brucine salts was also introduced by us.

Thannhauser first announced the isolation of a trinucleotide from the products of digestion of nucleic acid by enzymes. Later, Thannhauser and Dorfmüller hydrolyzed nucleic acid by means of 25 per cent ammonia and supposedly cleaved the molecule into uridinphosphoric acid and a trinucleotide containing the remaining three nucleotides. On acid hydrolysis with 2 per cent sulfuric acid these authors obtained only uridinphosphoric acid. This publication appeared in Germany in 1917 and did not reach us until 1919. In 1914, Jones and Richards described experiments by which they thought they had cleaved nucleic acid into two dinucleotides; guanin-cytosine, and adenin-uridin dinucleotides. Subsequently, Jones and his coworkers described the same two dinucleotides which they obtained on hydrolysis of yeast nucleic acid by heating the acid in an autoclave in a 2.5 per cent ammoniacal solution for 1½ hours at a temperature of 115°C. On the other hand, on hydrolysis by means of dilute acids, Jones and Read described a cytidin-uracil dinucleotide. On the basis of their respective findings, Jones and Thannhauser presented theories of the mode of linkage between the nucleotides.

According to Jones the structure of yeast nucleic acid is as follows:

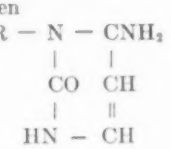


Thannhauser, on the other hand, presents the linkage of the nucleotides in the following way:[7]

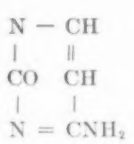


[7] In Thannhauser's representation there is an oversight in regard to the cytidin linking.

It is given



It should be



These theories are based on the following considerations:

That of Jones on the assumption of the reality of the dinucleotides, and on the observations that the so called dinucleotides are tetra-basic. The theory of Thannhauser is based on the assumption of the belief in the reality of the trinucleotide, and second, on the fact that the so called trinucleotide is hexo-basic.

In previous communications we have criticized the conclusions of these writers, on the assumption that their observations were correct.[8] In a later communication,[9] we have shown that the cytidin-uridin dinucleotide was a mixture of uridin and cytidin mononucleotides. Uridinphosphoric acid was obtained as a crystalline barium salt. It may be mentioned here that optical rotation of the crystalline salt air-dry was $[\alpha]_D^{20} = 3.5$, or dry and barium-free $[\alpha]_D^{20} = 5.83$, whereas Thannhauser and Dorfmüller found for their uridinphosphoric acid + 14.4.

On the other hand the barium salt of the cytidinphosphoric acid had the optical rotation of $[\alpha]_D^{20} = +14.0$ or barium-free and dry $[\alpha]_D^{20} = +23.3$ which agrees with the recent finding of Thannhauser for the crystalline cytidinphosphoric acid, which was $[\alpha]_D^{20} = 23$. Thus it is possible that the substance described by Thannhauser as uridinphosphoric acid was of a lesser degree of purity than that of the cytidinphosphoric acid.

In a still later publication[10] we reported on the finding that the so called cytidin-uridin dinucleotide was fractionated by us into uridinphosphoric and adenosinphosphoric acids. The former was identified as the crystalline barium salt, the latter as the brucine salt, which at the time of that publication was converted into the barium salt. Since from a large quantity of brucine salt there was obtained only a small quantity of a barium salt analyzing satisfactorily for the salt of the adenosin nucleotide, the publication of the analytical data on that nucleotide was delayed. It was subsequently found that adenosinphosphoric acid is identified most conveniently as the free acid. Crystalline adenosinphosphoric acid was described by Jones and Kennedy;[11] the substance obtained by us differed from that of

[8] Levene, P. A., *J. Biol. Chem.*, 1917, xxxi, 591.

[9] Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 21.

[10] Levene, P. A., *J. Biol. Chem.*, 1918, xxxiii, 425.

[11] Jones, W., and Kennedy, R. P., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 45.

Jones only in the fact that our material dried in air contained no crystal water, whereas the substance of Jones contained one crystal water. The optical rotation of our substance is $[\alpha]_D^{20} = -38.5$. Whereas the rotation of adenosinphosphoric acid remains constant either in water or in 5 per cent ammonia water, the rotation of guanosinphosphoric acid shows a marked increase in its levorotation in ammonia water. This point may serve for differentiation between the two nucleotides. Thus it is proven that the adenin-uridin dinucleotide is a mixture of two mononucleotides.

The fraction which was originally regarded by Jones as a guanin-cytosine dinucleotide, and from which Read isolated an amorphous guanylic acid, was also found by us to consist principally of guanosinphosphoric acid. In addition a small proportion of uridinphosphoric acid was found in this fraction. The presence of the latter nucleotide might have escaped isolation if not for its recently described property of forming a crystalline lead salt.

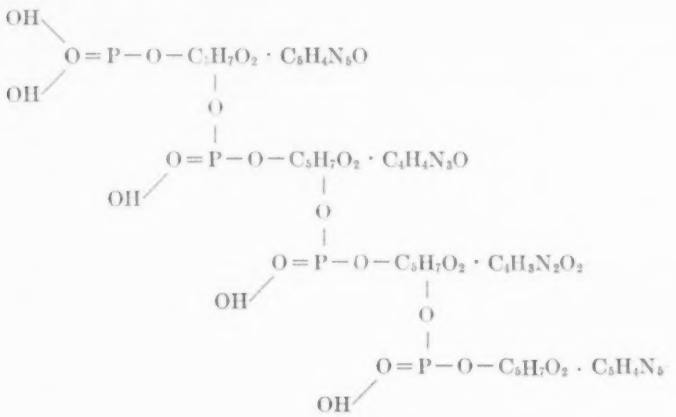
The guanylic acid was isolated in a crystalline form, and constituted the greater part of the fraction.

The method of hydrolysis employed by us consisted in heating the nucleic acid in a 2.5 per cent aqueous ammonia solution for 1 hour at 100°C. Thus the treatment was milder than the one employed by either Jones or Thannhauser. Thus the present findings nullify the experimental evidence in support of the theories of Jones and of Thannhauser. From the theoretical point of view, the theory of Thannhauser is not very tenable for the reason that a carbon to carbon-linking implies a very strong union, whereas the polynucleotide is readily dismembered into mononucleotides. Thannhauser, in fact, accepts it himself with great reserve. As regards the ether-linking accepted by Jones, it must be remarked that, as a rule, an ether-linking represents a very firm union. If one accepts that this rule does not apply to carbohydrates linked in ether form, he should present experimental evidence in support of this view.

However, if the work of Jones and of Thannhauser failed to support their speculations regarding the mode of linkage of the mononucleotides, it has been of great importance in furnishing further proof of the nucleotide structure of yeast nucleic acid;

and also, in making it possible to show that the molecule of nucleic acid is readily decomposed into mononucleotides, and that the linkage between all nucleotides is of the same order.

On the basis of considerations such as these the linkage of the nucleotides could be expressed most simply in the following way:



For the present this form expresses the facts known about the structure of yeast nucleic acid. New facts and new evidence may cause its alteration, but there is no doubt as to the polynucleotide structure of the yeast nucleic acid.

It is unfortunate that, owing to war conditions, the work of Thannhauser was not known to us earlier, also that apparently our work was not known to Thannhauser.

EXPERIMENTAL.

The mode of hydrolysis was practically the same as that described in a previous communication,[10] with a difference in one detail; namely, the temperature of the autoclave was maintained at 100°C.

Treatment of the product of hydrolysis was also the same as described in that communication, and essentially the same as employed by Jones and his collaborators. The fraction precipitated by 98 per cent alcohol will be referred to as guanin fraction, and that remaining in solution as adenin fraction.

Adenin fraction was treated in exactly the same manner as that described in the previous communication. The brucine salt was recrystallized nine times with boiling 35 per cent alcohol.

The crystalline deposit consisted of uridinphosphoric acid previously described. The first three mother liquors, on concentration, gave a brucine salt containing C = 53.00, H = 6.40, and N = 10 per cent. The subsequent six mother liquors, on concentration, gave a brucine salt containing N = 8.5 per cent.

The brucine salt of the first three mother liquors was transferred into the ammonium salt. Originally the ammonium salts were converted into the barium salt. Barium salts, having analytical value sufficiently approaching that required by the theory, were obtained only after many purifications which were associated with much loss. Finally, an attempt was made to transform the ammonium salt into the free nucleotide. This was accomplished without difficulty in the following manner. To the hot solution of the ammonium salt, while the mixture was agitated, a hot solution of neutral lead acetate was added in a slow stream. When the necessary volume of lead acetate (25 per cent solution) was added, the mixture was brought to a boil and filtered. The precipitate was washed in a mortar and filtered; the operation was repeated three times. Finally the precipitate was suspended in water, treated with hydrogen sulfide, and the filtrate from lead sulfide was concentrated under diminished pressure at room temperature. On standing, adenosinphosphoric acid crystallized in long needles resembling the free nucleoside. The substance differed from that described by Jones in that it crystallized without crystal water. The analysis of the air-dry substance was as follows:

0.1010 gm. of the substance gave 0.1268 gm. of CO_2 and 0.0368 gm. of H_2O.

0.1000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 14.26 cc. of 0.1 N acid.

0.3000 gm. of the substance gave 0.0940 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5PO_7$. *per cent*	Found. *per cent*
C	34.57	34.24
H	4.07	4.08
N	20.17	19.96
P	8.94	8.73

The optical rotation of the substance in aqueous solution was

$$[\alpha]_D^{20} = \frac{-0.77 \times 100}{1 \times 2} = -38.5$$

In a solution of 5 per cent ammonia water the rotation was

$$[\alpha]_D^{20} = \frac{-0.80 \times 100}{1 \times 2} = -40.0$$

Hydrolysis of the Adenosinphosphoric Acid.—2 gm. of the substance in 35 cc. of 1 per cent sulfuric acid were boiled over flame with reflux condenser for 1 hour. The product of hydrolysis was neutralized with sodium hydroxide and to the neutral solution aqueous picric acid was added as long as a precipitate formed. The precipitate was dissolved in hot water and allowed to crystallize.

The analysis of the air-dry substance was as follows:

0.1000 gm. of the substance gave 26.2 cc. of nitrogen gas at $T° = 26°C.$ and $P = 752$ mm.

	Calculated for $C_5H_5N_5.C_6H_2(OH)(NO_2)_3+H_2O$.	Found.
	per cent	*per cent*
N	29.32	29.60

The substance decomposed at 177°C. (uncorrected).

Brucine Salt of Adenosinphosphoric Acid.—2 gm. of the nucleotide were dissolved in hot water and the solution was neutralized with a solution of brucine in methyl alcohol. On cooling, the solution nearly solidified. The crystals of the brucine salt of the nucleotide were filtered off with suction, and the substance was recrystallized three times out of 35 per cent alcohol.

The air-dry substance on heating in a sealed capillary tube melted as follows: At 177°C. it began slightly to contract; at 195°, the substance effervesced, remaining perfectly colorless; at 225° a second point of effervescence was observed, the substance turning dark. The substance analyzed as follows:

0.1020 gm. of the substance gave 0.1978 gm. of CO_2 and 0.0572 gm. of H_2O.

0.2000 gm. of the substance gave 17.6 cc. of nitrogen gas at $T° = 24°C.$, $P = 769$ mm.

0.3000 gm. of the substance gave 0.0244 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5PO_7.(C_{20}H_{25}O_4N_2)_2 \cdot 7H_2O$.	Found.
	per cent	*per cent*
C	53.28	52.88
H	6.40	6.28
N	10.00	10.23
P	2.47	2.27

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.74 \times 100}{1 \times 2} = -37\ 0$$

The guanin fraction consisted principally of guanosinphosphoric acid (guanylic acid). It was treated in a general way in the manner indicated by Read. The lead salts were converted into the brucine salts, and these were fractionated in the same manner as the salts of the adenin fraction. However, the substance obtained from all the nine mother liquors contained over 10 per cent of nitrogen and only the fraction constituting the ultimate crystalline deposit contained on analysis about 8.75 per cent of nitrogen. Surprisingly also this fraction consisted in the main of guanylic acid. The brucine salts were converted into the ammonium salts. These were dissolved in boiling water and to the hot solution a hot solution of lead acetate was added in a slow stream. The mixture was then brought to a boil and filtered hot. The lead precipitate was freed from lead and concentrated under diminished pressure at room temperature. Generally an amorphous, somewhat gelatinous precipitate settles out. In some instances the solution turns into a semiliquid jelly. To bring about final crystallization, no general rule can be given. At times repeated precipitation with lead acetate will lead to a filtrate which, on concentration, solidifies into a crystalline mass. Often it is advisable to precipitate the nucleotide by means of lead acetate fractionally. The later fractions as a rule crystallize with less difficulty.

The properties and analysis of the crystalline guanylic acid were described in a previous communication.[12]

When the brucine salts with 8.75 per cent of nitrogen were converted into ammonium salts, and when these were taken up

[12] Levene, P. A., *J. Biol. Chem.*, 1919, xl, 171.

in hot water, part of the substance remained insoluble. This residue consisted of brucine salt which escaped, being converted into ammonium salt. The brucine salt on analysis showed a nitrogen content of N = 7.8 per cent. This brucine salt was then converted into the ammonium salt. The latter was dissolved in boiling water, and a hot solution of neutral lead acetate was added. The mixture was brought to a boil and filtered hot. The filtrate was seeded with a few crystals of the lead salt of uridinphosphoric acid, and allowed to stand near a hot water bath. It was found that when the cooling of the filtrate proceeded rapidly a gelatinous lead salt settled out. If, however, the cooling was progressing slowly the lead salt of uridinphosphoric acid settled out in crystalline form. For analysis the substance was dried to constant weight under diminished pressure at the temperature of xylene vapor. It analyzed as follows:

0.1118 gm. of the substance gave 0.0856 gm. of CO_2 and 0.0218 gm. of H_2O.

0.1848 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 7.7 cc. of 0.1 N acid.

0.2772 gm. of the substance gave 0.0574 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{11}N_2PO_9Pb$.	Found.
	per cent	*per cent*
C	20.40	20.73
H	2.10	2.18
N	5.29	5.84
P	5.86	5.78

THE ACTIVITY OF LUNG EXTRACT, AS COMPARED TO EXTRACTS OF OTHER TISSUES, IN INDUCING COAGULATION OF THE BLOOD.

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(From the Department of Biochemistry, University of Cincinnati, Cincinnati.)

(Received for publication, October 14, 1919.)

Wherry and Ervin (1), in experimenting with the intravenous injection of extracts of tuberculous lung tissue, found that very small doses of an extract of normal as well as tuberculous lung tissue would produce death in a few seconds in rabbits and guinea pigs. It was at their request that I undertook to discover the cause of the sudden death in these cases.

The extracts were made by grinding the fresh lung tissue well with sand in a mortar, adding gradually while stirring 10 cc. of 0.9 per cent NaCl for each gm. of tissue taken. The mixture was then centrifuged for 20 to 30 minutes at about 3,000 revolutions per minute and the slightly cloudy, reddish solution used for the injections. It was found by trial that 0.3 cc. of this solution injected *rapidly* into the ear vein of a rabbit weighing 1,000 to 1,500 gm. caused respiratory symptoms of irregular breathing and uneasiness in 20 to 30 seconds, weakness and prostration shortly afterwards, and death with convulsions and respiratory spasms usually within 1 minute after the injection. If a larger dose, such as 0.5 cc., was injected rapidly into a rabbit of this size respiratory symptoms began in 20 seconds and death, accompanied by violent convulsions and spasms, followed within 10 seconds. For rabbits of 1,800 to 2,500 gm. the dose necessary to produce death was 0.4 cc. so that the reaction is in a measure quantitative.

On examination of the rabbits immediately after cessation of the spasms, the heart was usually found beating rhymically, although there were sometimes arrhythmias and fibrillation. All organs appeared normal, the lungs always being found collapsed

after opening the thorax. On opening the heart or vessels, larger or smaller clots were always found and sometimes the whole blood was found to be coagulated, especially if more than the minimum fatal dose was given. In cases where the whole blood was not clotted, the escaping fluid portion clotted more slowly than normal, the length of time before the occurrence of spontaneous clotting varying from a few minutes to a day.

These observations lead to the conclusion that in these cases death was due in a large measure at least to intravascular clotting, the symptoms probably resulting from the complete asphyxia of the nervous system. Although the death resembled that of anaphylaxis, yet in contradistinction to the latter the lungs were always found to collapse on opening the thorax, whereas in deaths from anaphylaxis the lungs do not collapse.

I therefore undertook the study of the particular power of lung tissue in causing thrombosis, as I was unable to find any reference to the peculiar toxicity of lung extracts in this respect. This study has resulted in showing the very remarkable thromboplastic power of the lungs, a power far surpassing that of any other tissue of the body.

Intravascular coagulation from the intravenous injection of tissue extracts has been known to be possible for many years. Quoting from Carpenter (2),

> "The contact of dead animal matter with the blood appears to promote the coagulation of its fibrin in a very remarkable degree; occasioning coagula to form, whilst it is yet actively moving in the vessels of the living body. Thus M. Dupuy found that the injection of cerebral substance into the veins of an animal occasioned its death almost as instantaneously as if prussic acid had been administered; the circulation being rapidly brought to a stand, by the formation of voluminous clots in the heart and large vessels. These experiments were repeated and confirmed by M. de Blainville. (Gazette Medicale, 1834, p. 521.) The same effect is produced with still more potency, when the substance injected is rather undergoing degradation, than actually dead; for it then seems to act somewhat after the manner of a ferment, producing a marked diminution in the vitality of the solids and fluids with which it may be brought in contact. Such is pre-eminently the case with *pus*, as was long ago observed by Hunter and as Mr. H. Lee has since determined more precisely. It was found by the latter, that healthy blood received into a cup containing some offensive pus coagulated in *two* minutes; whilst another sample of the same blood, received into a clean vessel of similar size and shape, required *fifteen* minutes for its complete coagulation."

Wooldridge (3), working from 1881 to 1889, was the first, however, to attempt to isolate the active thromboplastic material from the tissue extracts and to make a thorough study of the action of such extracts. He used extracts prepared from testes, thymus, and lymph glands, extracting the fresh tissues with water and precipitating the active material from the solution by making it strongly acid with acetic acid. The precipitate was then washed in water and dissolved in very dilute carbonate solution. Such solutions he found to produce thrombosis throughout the whole vascular system of rabbits after rapid intravenous injections, but in dogs, clots were usually found only in the portal system. In any case, if the animal survived the thrombosis, a second injection within 24 hours was without effect, and blood drawn after such injection had a greatly diminished coagulability, spontaneous coagulation often being delayed as long as 24 hours. The remarkable fact was discovered that the addition of more of the tissue extract to this blood outside the body produced coagulation in a few minutes. He observed that the degree of non-coagulability of the blood was in a measure proportional to the extent of the thrombosis.

In studying the extracts to determine the active substance, he decided that a protein-phospholipin compound was responsible for the results. This compound could be extracted from the acetic acid precipitate with dilute alkali. He states that the solution was not a true solution since the dissolved substance would not pass through a clay cell. If he extracted this precipitate with alcohol and ether the activity of the undissolved residue was lost, so that the phospholipin must be a necessary part of the compound. Also on digestion of the solution with pepsin and hydrochloric acid, the phospholipin, with a small amount of the protein, was precipitated, and the remaining solution had lost its activity. The precipitate was active. Examination of the phospholipin convinced him it was a lecithin-like substance, although purified lecithin from egg yolk was not active. Lecithin prepared from other tissues gave him the same results as the tissue extracts. Wooldridge was not familiar with cephalin at that time, so that he did not identify the active phospholipins as cephalin which was present as an impurity in his tissue lecithin, although he did show that not all lecithin prepa-

rations were active. More recent work in Howell's laboratory (4) indicated that the phospholipin inducing coagulation discovered by Wooldridge is cephalin, but the strange fact appeared from my experiments that although the nervous tissue is the tissue believed to be the richest in cephalin, yet extracts of the brain made in the same way as these lung extracts did not cause intravascular coagulation and death in rabbits in doses up to 3 cc. for an 1,800 gm. rabbit. It was therefore deemed advisable to compare as quantitatively as possible the thromboplastic activity of lung tissue extracts with that of extracts of other tissues of the body.

Fresh tissues of dogs killed in ether anesthesia and rabbits were used in making extracts similar to the lung extract described above, that is, for every gm. of the fresh tissue, after grinding thoroughly with sand, 10 cc. of 0.9 per cent NaCl solution were added, mixed well, and centrifuged to remove all solid particles. Extracts thus made were found to undergo a gradual loss of their power to induce coagulation when injected into the circulation or of hastening coagulation when added to blood outside the body. When standing at 5°C. very little change occurred in the first few days, but activity had almost disappeared at the end of 3 weeks. This loss of activity was not due to a settling out of the active matter formerly in suspension, since on shaking and injecting, or adding precipitate and solution mixed, the diminution of activity persisted. It might be, however, that a gradual agglomeration of particles had occurred leading to a smaller number of larger aggregates with a resulting diminution of surface and hence of activity. This rather than a chemical change might explain the progressive loss of power. This possibility will be further investigated.

The activity of the tissue extracts prepared as described were tested in two ways, (1) by injections into the blood stream of dogs and rabbits, and (2) by testing their power of hastening coagulation when added to peptone and oxalate plasma outside the body. In the latter case the test-tube method for determining the coagulation time was used, coagulation being considered complete when the tube could be inverted without spilling the contents. If care was taken in shaking the tubes and in keeping all other factors constant, this method was considered sufficiently accurate for this work.

Peptone plasma and oxalated plasma of dog's blood were used in most of the tests, although two sets of rabbit tissue extracts were tested on rabbit blood rendered partially non-coagulable by a process which will be described in another paper.

In order to compare the activity of the extracts of the different tissues, one method was to find the amount of the various extracts necessary to induce coagulation of 1 cc. of peptone plasma in the same time as a definite amount of lung extract. Another method used was to add the same amount of the different extracts to oxalate plasma and compare the amount of acceleration of coagulation of the plasma by serum. All tests were carried out in a water bath at 38–40°C.

Tables I and II indicate the results of the tests carried out with the extracts of the tissues of two different dogs.

The pancreas, skeletal muscle, thyroid, and omentum contained so little of the thromboplastic material that they would not accelerate the coagulation to 30 seconds in any amount, so the figures for them are approximations derived from the degree of lessening of coagulation time by increasing amounts of the extracts.

TABLE I.

Tissue extract used (dog).	Time of coagulation of 1 cc. of oxalate dog plasma, by 3 drops of extract and 6 drops of serum.*	
	Tissues of Dog I.	Tissues of Dog II.
	min.	*min.*
Lung	½	½
Kidney	4	4
Testes	7	6
Brain	8½	6
Heart	8	7
Spleen	9	4
Bone marrow	7	7
Adrenal	8	8½
Liver	11	8
Omentum	9	16
Skeletal muscle	14	12
Pancreas	14	14
Thyroid	14	15

* 1 cc. of oxalate plasma and 6 drops of serum (with no extract) showed no coagulation in 1½ hours, but were coagulated in 24 hours.

TABLE II.

Tissue extract used (dog).	Amount of extract necessary to coagulate 1 cc. of peptone dog plasma in 30 seconds.	
	Tissues of Dog I.	Tissues of Dog II.
	gtt.	gtt.
Lung	2	2
Kidney	5	5
Testes	8	15
Brain	7	6
Heart	20	12
Spleen	4	4
Bone marrow	20	20
Adrenal	6	20
Liver	25	20
Omentum	30 +	30 +
Skeletal muscle	30 +	30 +
Pancreas	40 +	30 +
Thyroid	30 +	20 +

The activity of extracts of the tissues of two rabbits on partially non-coagulable rabbit blood is shown in Table III.

TABLE III.

Tissue extract used (rabbit).	Amount of extract necessary to clot 1 cc. of rabbit blood.	
	90 seconds. Tissues of Rabbit I.	70 seconds. Tissues of Rabbit II.
	gtt.	gtt.
Lung	1	1
Kidney	5	5
Heart	5	5
Thymus		3
Spleen	8	4
Brain	8	13
Skin		10
Testes		20 +
Ovary	22	
Uterus	20	
Liver	30 +	30
Bone marrow		20 +
Pancreas		20 +
Adrenal	26 +	20 +
Omentum	30	
Skeletal muscle	30 +	30 +

Here again some of the tissues were so poor in the active substance that they would not clot the blood in the specified time in any amount, so that the figures given are only approximations. *In every case studied the lung extract was found to be much stronger than that from any other tissue*, being from two to thirty times as strong as the other tissues in accelerating coagulation. Kidney, heart, brain, spleen, thymus, and skin come next in activity, somewhat in the order named. Then, certain other tissues, pancreas, skeletal muscle, liver, bone marrow, omentum, and adrenal, showed only slight thromboplastic activity as compared to the lung tissue.

Having thus established the predominant thromboplastic power of the lung extracts I next attempted to discover whether the toxicity from intravenous injections of these extracts paralleled their thromboplastic activity. To this end injections were made into rats and rabbits. In using rats the injections were made directly into the heart, but with rabbits injections were into an ear vein. Adult white rats, weighing about 350 gm., were used. They were found to be much less affected by partial coagulation of the blood in the vessels than rabbits, quick death being produced only by almost solid intravascular coagulation following injections of relatively large doses of the extracts. Most of the extracts of rabbits and rat tissues would not produce coagulation in rats in doses up to 3 cc. when made up in the usual way, so double strengths of these extracts were used, that is, only half the usual amount of saline solution was used for each gm. of fresh tissue. These extracts were labeled (×2). Table IV indicates the dosages of the various extracts necessary to produce rather extensive coagulation in the vessels of the rats.

In intravenous injections into rabbits only two of the tissue extracts proved fatal, death occurring in rabbits after only slight thrombosis. Lung extract was fatal in doses of 0.3 to 0.4 cc. and kidney extract (×2) was fatal in a dose of 2.0 cc. producing only a slight amount of thrombosis, and with death occurring after a delay of 2½ minutes. None of the other tissue extracts was fatal in doses up to 3.0 cc. Wooldridge reports solid thrombosis of the whole system in rabbits with injections of thymus, testes, and lymph gland extracts, but he used many times the amount of material for injections that was used in these cases.

Thus he used ½ to 1 gm. of the washed acetic acid precipitate from the extracts to obtain such solid intravascular coagulations.

From these intravenous injections into rats and rabbits as described above, it is evident that the extracts are toxic in much the same order as they exhibit thromboplastic activity in extravascular coagulation, lung extract being much the most active of all tissue extracts in each case.

Most hemostatic preparations on the market today are prepared from brain material, but from the results of the above experiments it would seem that lung tissue would afford a much stronger hemostatic. Battelli (5) describes a method for preparing such material from fresh horse or sheep lungs.

TABLE IV.

Extract used.	Coagulative dosage for rats.
	cc.
Rat lung extract	0.5
" kidney "	1.0
" " " (×2)	0.9
" brain " (×2)	1.1
Rabbit lung extract	0.4
" kidney " (×2)	0.9
" brain "	3.0
" " " (×2)	1.0
" heart " (×2)	1.5
" liver " (×2)	2.0

The significance of such a strong thromboplastic activity in lung tissue, and not so very much less in kidney tissue, is an interesting question. Is it a protective mechanism against possible hemorrhages in these organs in which the rich capillary network is covered only by a one celled layer of epithelium? This high activity of the lung tissue may perhaps have an especial function in certain diseases, such as pneumonia, where there occurs such an extensive destruction of lung tissue. With the extremely rich blood supply in all parts of the lungs one would expect severe hemorrhages in these cases, but the hemorrhages are, in fact, not one of the main characteristics of this disease. May it not be the liberation of this active material by the tissue destruction which protects against extensive hemorrhages? The

one extract of the skin tested was also found to possess considerable thromboplastic activity, again denoting a possible protective mechanism.

The investigation of the nature of the active principle in lung extract is being carried further and will form the subject of another paper.

SUMMARY.

1. Sudden death from the intravenous injection of lung tissue extract is apparently due to intravascular coagulation, although there is a possibility of anaphylactic effect on the lungs, not sufficient, however, to keep them from collapsing on opening the thorax.

2. Tissue extracts were tested as to their thromboplastic activity on the blood both intra- and extravascularly. Lung extracts were found far more active than the extracts of any other tissue, kidney coming second, and then heart, brain, spleen, thymus, testes, skin, somewhat in the order named. The remaining tissues were weakly active as compared to lung, some of them showing very slight thromboplastic action.

3. Lung tissue offers a possible source for the preparation of a strong hemostatic.

4. The strong coagulative activity of lung and kidney tissues, and to a lesser degree of skin, is suggestive of a possible protective mechanism against hemorrhage.

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THE HYDROLYSIS OF STIZOLOBIN, THE GLOBULIN OF THE CHINESE VELVET BEAN, STIZOLOBIUM NIVEUM.

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(Received for publication, October 28, 1919.)

The preparation of the globulin used for this hydrolysis is described in a previous publication from this laboratory (1).

The hydrolysis and the determination of the resulting amino-acids were carried out, for the greater part, in the usual manner, with a few exceptions which will be noted later.

A slight modification of the customary method of drying the ether solutions of the esters with anhydrous sodium sulfate was made. It seems very probable that during the extraction of the esters with ether, after their liberation with sodium ethylate, or with sodium hydroxide, as is sometimes necessary after the second esterification, some of the alkali passes into the ether, and later decomposes to some extent the easily hydrolyzed esters. Accordingly, finely powdered ammonium chloride was mixed with the sodium sulfate used for drying the ether solution of the esters. Any caustic alkali present would thus be removed by the action of ammonium chloride, with the formation of sodium chloride and ammonia, and in this way the hydrolysis of the esters reduced to a minimum.

The method of Levene and Van Slyke (2) for the separation of alanine from valine by means of phosphotungstic acid was used with good results.

The percentages of basic amino-acids in stizolobin, as determined by the Van Slyke method, have already been published (1).

The percentages of the various amino-acids obtained by the hydrolysis of stizolobin are summarized in Table I. For the sake of comparison, the percentages of amino-acids found in phaseolin,

the globulin of the white navy bean, *Phaseolus vulgaris*, are also given.

As is seen, the summation of the hydrolysis of stizolobin, although comparatively high, falls far short of accounting for the theoretical amount of hydrolysis products which should result. Some time ago we became convinced that with the methods now in use one cannot hope to account quantitatively for nearly all

TABLE I.

Percentage of Amino-Acids in Stizolobin and Phaseolin.

	Stizolobin.	Phaseolin.*
	per cent	*per cent*
Glycine	1.66	0.6
Alanine	2.41	1.8
Valine	2.88	1.0
Leucine	9.02	9.7
Proline	4.00	2.8
Phenylalanine	3.10	3.3
Aspartic acid	9.23	5.3
Glutaminic acid	14.59	14.6
Hydroxyglutaminic acid	2.81	
Serine	0.67	0.4
Tyrosine	6.24†	2.2
Cystine	1.13	
Arginine	7.14	4.9
Histidine	2.27	2.0
Lysine	8.51	4.0
Tryptophane	Present.	
Ammonia	1.55	2.1
Total	77.21	54.7

* Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1907, xviii, 295.
† By the colorimetric method of Folin and Denis.

the products which proteins yield on hydrolysis, but that methods must be developed whereby the products of hydrolysis can be separated more directly than by esterifying the amino-acids and distilling the esters as originally worked out by Fischer. As is well known, it is rarely if ever a 100 per cent esterification of a pure organic acid can be accomplished, and when one considers the complex mixture which results from the hydrolysis of a protein, consisting of eighteen or more amino-acids, humin, and other

secondary decomposition products, a high percentage esterification can hardly be expected. The hope of attaining anywhere near a theoretically complete summation is therefore practically excluded from the beginning. We have been working for some time on the direct determination of the amino-acids in another protein by methods which do not involve the process of esterification and distillation of esters. This work is still in progress.

A method for the determination of proline without involving the esterification of the amino-acids is described. The products of hydrolysis of the protein, after having first removed the bases and mineral acids, are obtained in a dry powdered form, from which the proline is extracted with boiling absolute alcohol. The non-amino nitrogen in the solution is determined and the per cent of proline calculated. Several determinations made in this way gave results closely agreeing with each other, and also with the percentage obtained from the esters.

Since the work on this hydrolysis was completed an article published by Dakin (3) appeared in which he describes a method for the partial separation of the amino-acids in a hydrolysis mixture by extracting the aqueous solution of amino-acids with butyl alcohol, and in which is also described for the first time the isolation and properties of the new amino-acid, hydroxyglutaminic acid. By means of this method, Dakin states that the products of hydrolysis of a protein may be readily separated almost completely into the following five groups:

(1) Monoamino-acids, both aliphatic and aromatic, insoluble in alcohol but extracted by butyl alcohol.

(2) Proline, soluble in alcohol and extracted by butyl alcohol.

(3) Peptide anhydrides (diketopiperazines), extracted by butyl alcohol but separated from (2) by sparing solubility in alcohol or water.

(4) Dicarboxylic acids, not extracted by butyl alcohol.

(5) Diamino-acids, not extracted by butyl alcohol but separable from (4) by phosphotungstic acid and other means.

In order to check up by this method the results we had already obtained for aspartic and glutaminic acids in stizolobin by the ester method, as well as to look for hydroxyglutaminic acid, some of the residues of amino-acids remaining from the direct determination of proline were examined by this method for

aspartic, glutaminic, and hydroxyglutaminic acids. 2.81 per cent of hydroxyglutaminic acid was obtained in the form of the difficultly soluble silver salt.

One of the most striking results of this examination is the extraordinarily high percentage of aspartic acid obtained; namely, 9.23 per cent. As the amount obtained from the esters was only 5.70 per cent, it is highly indicative that the percentages of aspartic acid obtained in previous hydrolyses of proteins do not nearly represent the actual amount present.

The amount of glutaminic acid obtained by this method was somewhat lower than that obtained from the large hydrolysis, due to the fact that some of this amino-acid had been previously removed in the form of pyrrolidonecarboxylic acid, while making the proline determination.

The method outlined by Dakin will doubtless prove of great value in the further development of methods for the direct separation and determination of the products of hydrolysis of proteins.

EXPERIMENTAL.

A quantity of stizolobin, equivalent to 405 gm. of the ash and moisture-free protein, was hydrolyzed in two separate portions of 200 and 240 gm. each, with 1,300 cc. of hydrochloric acid (specific gravity 1.1), by first heating on a steam bath until nearly all the protein had dissolved. The hydrolysis was then continued by boiling the solutions in an oil bath for 30 hours. The solutions were then concentrated to about one-half their original volumes, and after removal of most of the color by treatment with norite, were saturated, cold, with dry hydrochloric acid gas. After standing for about a week at nearly 0°, the glutaminic acid hydrochloride was removed by filtration. There were finally obtained, after removal of ammonia with barium hydroxide in the usual way, 27.23 and 24.83 gm., respectively, of glutaminic acid hydrochloride, equivalent to a total of 41.72 gm. of the free acid. This amount, together with the 17.28 gm. subsequently isolated from the esters, is equivalent to 14.59 per cent of the stizolobin.

The glutaminic acid hydrochloride decomposed with effervescence at 197°. The free acid, obtained by decomposing the

hydrochloride with an equivalent amount of normal potassium hydroxide, decomposed at 202° and was analyzed with the following results:

0.1491 gm. of substance gave 0.2249 gm. of carbon dioxide and 0.0872 gm. of water.

	Calculated for $C_5H_9O_4N$.	Found.
C	40.80	41.14
H	6.12	6.54

The filtrates and washings from the above direct determination of glutaminic acid hydrochloride were united and concentrated to a heavy syrup. This residue was freed from water by repeated evaporations with alcohol under reduced pressure and esterified in the usual way, with the exception that no zinc chloride was used. After liberating the esters with sodium ethylate the sodium chloride was removed by centrifugation and the alcohol distilled from the esters under reduced pressure. The alcohol was reserved for further examination. On stirring the syrupy residue of esters with ether nearly all dissolved, but on dilution with more ether a flocculent precipitate separated which soon settled and formed a sticky, viscous layer. The addition of ether was continued until only a slight turbidity was produced. The ether extracts were filtered and dried in the usual way over anhydrous sodium sulfate.

The sodium chloride obtained from the alcoholic solution of the esters was dissolved in water and the solution saturated with hydrochloric acid gas. The salt which separated was removed by filtration. To the filtrate was added the above mentioned sticky residue which did not dissolve in the ether when extracting the esters. The solution was then subjected to a second esterification. The residue of ester hydrochlorides left after removing the alcohol by distillation was very viscous. It was therefore dissolved in enough absolute alcohol to form a thin syrup. After cooling to 0° the esters were liberated by the cautious addition of an aqueous 50 per cent solution of sodium hydroxide. The esters were extracted with ether and dried over anhydrous sodium sulfate to which was added a little powdered ammonium chloride.

The ether was removed from the esters by distillation at atmospheric pressure. 332 gm. of esters were obtained, 128 gm. of which were obtained from the second esterification.

Alcohol Distilled from the Esters.—The alcohol which had been distilled from the esters was acidified with hydrochloric acid and the solution evaporated to dryness under reduced pressure. The residue of ester hydrochlorides was hydrolyzed by boiling with dilute hydrochloric acid, and the solution evaporated to dryness. After expelling ammonia by boiling with barium hydroxide, and subsequent removal of barium and hydrochloric acid, there were obtained 8.48 gm. of amino-acids. The amino-acids insoluble in absolute alcohol weighed 6.66 gm. Both the proline and the other amino-acids were added to the corresponding portions of Fraction I of the distilled esters.

Ether Distilled from the Esters.—The ether distilled from the esters was strongly acidified with a dry alcoholic solution of hydrochloric acid, and allowed to stand at nearly 0° for several weeks. The white crystalline product which separated was boiled with barium hydroxide to remove ammonia. The barium and hydrochloric acid were then quantitatively removed, and after concentrating the solution to a small volume 3.81 gm. of glycine separated, which, for identification, was converted into its ethyl ester hydrochloride. The latter crystallized from alcohol in the characteristic long prisms or needles which melted sharply to a clear oil at 144°.

0.1987 gm. of substance required 14.15 cc. of 0.1 N sulfuric acid.

	Calculated for $C_4H_{10}O_2NCl$.	Found.
N	10.04	10.03

The glycine thus obtained, together with that subsequently isolated from the distilled esters, amounted to 6.72 gm. or 1.66 per cent of the protein.

The amino-acids present in the form of their esters in the original ether filtrate from the glycine ester hydrochloride were regenerated and weighed 9.24 gm., of which 6.88 gm. were insoluble in absolute alcohol. The latter and the alcoholic extract of proline were added to similar portions of Fraction I of the distilled esters.

The esters obtained after distilling off the ether were separated into the following fractions in the usual way by distillation under reduced pressure:

Fraction.	Temperature of the bath.	Temperature of the vapors.	Pressure.	Weight.
	°C.	°C.	mm.	gm.
I	100	50	35	21
II	113	85	3	85
III	135	107	3	61
Distillation residue				131
Contents of the liquid air tube				10

The contents of the liquid air tube were made strongly acid with hydrochloric acid and hydrolyzed by boiling for several hours. After expelling the ammonia with barium hydroxide and subsequent removal of barium and hydrochloric acid there were obtained 2.87 gm. of amino-acids, of which 2.04 gm. were insoluble in absolute alcohol. The latter were added to Fraction I.

Fraction I.—The esters of this fraction, which also contained some alcohol, were hydrolyzed in the usual way by boiling with water. There were obtained 4.64 gm. of amino-acids, 3.73 gm. of which were insoluble in absolute alcohol. To the latter were added the alcohol-insoluble amino-acids obtained from the following sources: (1) The alcohol distilled from the esters, 6.66 gm.; (2) the ether distilled from the esters, 6.88 gm.; (3) the contents of the liquid air tube, 2.04 gm.; (4) the precipitate which separated on standing from the united alcoholic extracts of proline, 2.30 gm. From this mixture were isolated by means of the lead salts, 5.62 gm. of leucine and 2.64 gm. of valine. The leucine had the following composition:

0.1825 gm. of substance gave 0.3678 gm. of carbon dioxide and 0.1671 gm. of water.

	Calculated for $C_6H_{13}O_2N$.	Found.
C	54.96	54.96
H	9.99	10.24

Analysis of the valine gave the following results:

0.1495 gm. of substance gave 0.2828 gm. of carbon dioxide and 0.1319 gm. of water.

	Calculated for $C_5H_{11}O_2N$.	Found.
C	51.28	51.59
H	9.47	9.80

The filtrates from the above leucine and valine were united, the amino-acids esterified and the resulting product was examined for glycine. There were obtained 5.0 gm. of glycine ester hydrochloride, equivalent to 2.69 gm. of glycine. The glycine ester hydrochloride crystallized from alcohol in the characteristic needles which melted sharply at 144°C.

The filtrate from the glycine ester hydrochloride, after having quantitatively removed the hydrochloric acid, yielded 7.37 gm. of alanine which crystallized in needles and prisms.

0.2028 gm. of substance gave 0.3004 gm. of carbon dioxide and 0.1377 gm. of water.

	Calculated for $C_5H_7O_2N$.	Found.
C	40.41	40.40
H	7.92	7.54

A small amount of glycine (0.22 gm.) was further separated (4) from the filtrate from the alanine, in the form of its picrate, which decomposed with effervescence at 195°C.

Fraction II.—This fraction yielded 52 gm. of amino-acids of which 42.6 gm. were insoluble in absolute alcohol. By direct fractional crystallization 16.50 gm. of leucine and 3.29 gm. of valine were obtained. Analysis of the leucine showed it to have the following composition:

0.1459 gm. of substance gave 0.2937 gm. of carbon dioxide and 0.1313 gm. of water.

	Calculated for $C_6H_{13}O_2N$.	Found.
C	54.96	54.90
H	9.99	9.99

The valine gave the following results on analysis:

0.1666 gm. of substance gave 0.3151 gm. of carbon dioxide and 0.1426 gm. of water.

	Calculated for $C_5H_{11}O_2N$.	Found.
C	51.28	51.58
H	9.47	9.51

A mixture of leucine and valine, after subjection to Levene and Van Slyke's lead salt method of separation, yielded 14.44 gm. of leucine and 2.43 gm. of valine.

A fraction was further obtained consisting of a mixture of alanine and valine. The amino-acids of this mixture were separated by means of phosphotungstic acid according to Levene and Van Slyke's method (2). 3.32 gm. of valine and 2.38 gm. of alanine were thus obtained.

All the alcoholic extracts of proline were united and allowed to stand for several days. The precipitate which had separated, after having been filtered off and washed with absolute alcohol, weighed 2.30 gm., and was added to the amino-acids of Fraction I. The clear solution was then evaporated to dryness under reduced pressure. The residue was completely soluble in cold absolute alcohol. The solution was again evaporated to dryness and the residue dissolved in water and made up to a volume of 500 cc. The total nitrogen in the solution was 2.6023 gm., and the amino nitrogen 0.7399 gm. The difference, or non-amino nitrogen, 1.8623 gm. corresponds to 15.30 gm. of proline, equivalent to 3.77 per cent of the proline. Special experiments for the direct determination of proline are described.

Fraction III.—Phenylalanine ester was separated from this fraction in the usual way by extraction with ether. After hydrolyzing the ester with hydrochloric acid, and decomposing the hydrochloride with ammonia, 4.16 gm. of phenylalanine were obtained.

0.1531 gm. of substance gave 0.3660 gm. of carbon dioxide and 0.0948 gm. of water.

	Calculated for $C_9H_{11}O_2N$.	Found.
C	65.45	65.20
H	6.66	6.88

The esters remaining after the removal of the phenylalanine were hydrolyzed with barium hydroxide, and yielded 2.92 gm. of aspartic acid as the barium salt, and 15.80 gm. in the form of the copper salt. The free aspartic acid gave the following results on analysis:

0.1664 gm. of substance gave 0.2200 gm. of carbon dioxide and 0.0825 gm. of water.

	Calculated for $C_4H_7O_4N$.	Found.
C	36.09	36.06
H	5.26	5.51

The copper aspartate was analyzed with the following results:

0.2503 gm. of substance (air-dried) gave 0.0717 gm. of copper oxide.

	Calculated for $C_4H_5O_4NCu \cdot 4\frac{1}{2}H_2O$.	Found.
Cu	23.07	22.90

There were also obtained 2.73 gm. of a substance which after several recrystallizations from water separated in the form of rather indefinite, microscopic, octagonal plates. Analysis showed these crystals to contain 35.20 per cent carbon, 7.00 per cent hydrogen, and 12.47 per cent nitrogen. The small amount of the substance in hand, and its high degree of solubility, prevented further examination of this material. Its solubility, crystalline form, sweetish taste, and composition, as above noted, leave little or no doubt that this substance was chiefly serine. Especially significant in supporting this conclusion is the high percentage of nitrogen found, as serine contains nearly 3 per cent more nitrogen than any of the other amino-acids usually found as products of protein hydrolysis, except proline. Serine theoretically contains 34.29 per cent carbon, 6.67 per cent hydrogen, and 13.33 per cent nitrogen.

Distillation Residue.—The residue remaining after the distillation of the esters was dissolved in water and shaken with ether to separate the phenylalanine, of which 8.41 gm. were obtained.

The aqueous layer was concentrated somewhat and sufficient concentrated hydrochloric acid added to make the solution about 25 per cent. This solution was boiled for 12 hours in order to hydrolyze any pyrrolidonecarboxylic acid that might be present. It was then concentrated to a small volume, and saturated cold with hydrochloric acid gas. After standing for a long time at 0°, 17.52 gm. of glutaminic acid hydrochloride were isolated. It decomposed with effervescence at 197°. The filtrates from the glutaminic acid hydrochloride were united and the bases removed with phosphotungstic acid. From the resulting solution, after removing the hydrochloric and phosphotungstic acids, there were further obtained 4.4 gm. of aspartic acid and 3.24 gm. of glutaminic acid.

Tyrosine.—Tyrosine was determined by the colorimetric method of Folin and Denis (5, 6). 1 gm. of stizolobin was boiled

for 12 hours with 20 per cent hydrochloric acid. The tyrosine was found to be equivalent to 6.24 per cent of the protein.

As the percentage of tyrosine obtained by this method is usually 2 or 3 per cent higher than that obtained by direct isolation of the amino-acid, due to the extreme difficulty of separating all the tyrosine from the other products of hydrolysis, a direct determination of tyrosine was made for comparison. 50 gm. of the protein were accordingly hydrolyzed with hydrochloric acid, and as much as possible of the latter was removed by distillation under reduced pressure. The calculated amount of normal potassium hydroxide to react with the chlorine remaining in the residue was then added. There were obtained from the resulting solution by careful fractional crystallization, 2.42 gm. of tyrosine, equivalent to 5.25 per cent of the protein. That not all the tyrosine was removed even then was indicated by the fact that the mother liquors still gave a strong positive test with Millon's reagent.

The tyrosine which was isolated gave the following results on analysis:

0.2036 gm. of substance required 11.2 cc. of 0.1 N HCl.

	Calculated for $C_9H_{11}O_3N$.	Found
N.....	7.73	7.72

Proline.—Several determinations of proline were made directly without involving the ester method of Fischer. The following procedure gave consistent results, which closely agreed with the percentage obtained by the ester method: 10 gm. of the protein are hydrolyzed by boiling with 20 per cent hydrochloric acid for about 30 hours. The solution is then concentrated under reduced pressure to a thick syrup, and the residue dissolved in water. After filtering off the suspended humin, the solution is made up to 200 cc. containing 3.5 per cent hydrochloric acid, and phosphotungstic acid added until all precipitation has ceased. After standing for 48 hours the precipitate is filtered off and washed in the usual way. Phosphotungstic acid is then removed from the filtrate by means of ether and amyl alcohol, and the solution of amino-acids boiled with a slight excess of barium hydroxide in order to remove any ammonia or phosphotungstic

acid that might be present. After filtering, the excess of barium is removed quantitatively with sulfuric acid and the solution made slightly alkaline with sodium hydroxide, in order to neutralize the hydrochloric acid present. The solution is then reacidified with acetic acid and evaporated to dryness, and the dry, finely powdered residue of amino-acids extracted by boiling with absolute alcohol for about 1½ hours. The alcoholic extract is acidified with hydrochloric acid and the alcohol removed by distillation under reduced pressure. The residue is then boiled for 10 to 15 hours with 25 per cent hydrochloric acid to regenerate any glutaminic acid from pyrrolidonecarboxylic acid which may have been formed during the preceding operations. The solution is then freed from hydrochloric acid as completely as possible by distillation under reduced pressure, the residue dissolved in water and made slightly alkaline with sodium hydroxide, and then reacidified with acetic acid. The resulting solution is made up to 100 cc. and amino nitrogen and total nitrogen are determined in 10 cc. aliquot portions. From these data the amount of proline is calculated. Eight determinations of proline made in this way gave closely agreeing results the average of which was 4 per cent.

The Di-Basic Amino-Acids.—As the residues of the amino-acids remaining from some of the direct proline determinations were on hand they were examined for the di-basic amino-acids according to the method of Dakin (3). Four of these residues, which represented 40 gm. of stizolobin, were united and used for the analysis. The proline, ammonia, bases, and most of the tyrosine had been previously removed. The amino-acids were dissolved in about 600 cc. of water and extracted with butyl alcohol, according to Dakin's directions. The extraction was continued for about 38 hours, at the end of which time there were practically no more acids being carried over by the butyl alcohol. As the amount of material available for this extraction was too small for a complete analysis, the monoamino-acids which had been carried over by the butyl alcohol were not further examined. The aqueous solution which had been extracted was examined for glutaminic acid in the usual way. 4.41 gm. of glutaminic acid, isolated as the hydrochloride, were obtained. This corresponds to 11.02 per cent of the protein. This percentage is somewhat lower than that which we obtained from

the main hydrolysis. This was to be expected, since some of the glutaminic acid was lost during the extractions for proline by being converted into pyrrolidonecarboxylic acid. The proline extract was always boiled with 25 per cent hydrochloric acid before determining amino nitrogen, and it was always found that some of the glutaminic acid had been converted into pyrrolidonecarboxylic acid.

The amino-acids in the filtrate from the glutaminic acid hydrochloride were converted into their calcium salts, according to Foreman's method (7), and the aspartic acid was isolated as the copper salt. An unusually large amount of the copper salt separated in the characteristic crystalline form. The air-dried salt weighed 7.69 gm., and without recrystallizing gave the following results on analysis:

0.2082 gm. of substance gave 0.0602 gm. of copper oxide.

	Calculated for $C_4H_5O_4NCu \cdot 4\frac{1}{2}H_2O$.	Found.
Cu	23.07	23.10

The above amount of copper aspartate is equivalent to 3.69 gm. of the free acid which is 9.23 per cent of the protein. This is 3.53 per cent more than was obtained from the esters.

Copper was removed from the filtrate from the copper aspartate, and the solution examined for hydroxyglutaminic acid by precipitating the acid in the form of its silver salt, according to Dakin's method. There were obtained 2.63 gm. of the difficultly soluble silver salt, which is equivalent to 1.12 gm. of hydroxyglutaminic acid, or 2.81 per cent of the protein.

0.2378 gm. of substance gave 0.1803 gm. of silver chloride.

	Calculated for $C_5H_7O_5NAg_2$.	Found.
Ag	57.26	57.07

SUMMARY.

The globulin of the Chinese velvet bean has been hydrolyzed and the percentages of the resulting amino-acids determined (see Table I).

A modification of the usual method of determining proline is described.

Aspartic acid was determined both by the usual ester method. and by the method, recently published by Dakin, of extraction of the solution of amino-acids with butyl alcohol. The yield obtained by the former method was 5.70 per cent, while by the latter method the unusually high percentage of 9.23 per cent was obtained. By means of the extraction method 2.81 per cent of hydroxyglutaminic acid was also obtained.

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THE NUTRITIVE VALUE OF THE BANANA. II.

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(Received for publication, October 10, 1919.)

Introduction and Object of the Investigation.

It has been shown in a preceding article[1] that bananas alone as a food do not produce growth of young albino rats. The addition of 16 per cent of purified casein, which supplied the protein deficiency of the food, and 0.5 per cent of yeast preparation, or of aqueous or alcoholic extract of fresh carrots, which supplemented the shortage of water-soluble accessory substance, constituted a complete diet for the growth, maintenance, and reproduction of albino rats. Such a diet was not adequate, however, for the production of proper milk by the mother. We stated that the addition of a small amount (10 cc.) of cow's milk to the mother's diet from the time of birth of the young until they are weaned is absolutely imperative, if the young are to survive and to maintain normal growth.

Further experiments on this point are shown in Tables I, II, and III, from which it is clearly seen that the new-born whose mothers were fed with a ration made up of bananas, casein, and yeast failed to grow and died from starvation, or were killed by the mother shortly after they were born, except in one instance where the young were reared but their body weights were very much below normal. The mother of these lost 10 gm. of her body weight during 1 month of lactation. On the other hand, the addition of cow's milk to the same diet made it a proper diet for the production of suitable milk during the period of lactation. The mothers maintained their body weights on this diet, and Rats 49 and 219 gained in body weight. The health and condition of these animals appeared normal in all respects.

[1] Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxvi, 171.

The aim of the present investigation has been to study the unknown chemical substance or substances present in milk, which are indispensable for the growth of young animals during the period of lactation.

Review of Previous Investigations.

Of all foods, milk is the most important. It contains all the nutrients essential for growth. The important constituents of milk are water, fats, casein,[2] lactalbumin,[3,4] lactoglobulin,[4,5] lactose, lecithin,[6] cholesterol,[7] urea,[8] ammonia,[9] the purine bases, alcohol-soluble protein,[10] inorganic salts, enzymes, and unidentified accessory factors.[11,12] Many foreign substances, such as flavors, condiments, and stimulants, introduced with the food, are secreted in the milk.

A brief review of the more characteristic investigations relating to milk production of adult animals and to the subsequent growth of young may throw light on the nature of the catalytic substances present in milk.

Decaisne,[13] during the siege of Paris, made an observation upon the milk production of forty-three women and the behavior of their infants. Twelve strong, healthy women had plenty of milk of good quality and their children obtained enough milk at the expense of catabolized tissue of the mothers. Fifteen women had little milk of poor quality. Their children became weak and had enteritis. Sixteen women had very little milk and more than three-fourths of the children died from starvation. Most of the forty-three women appeared to be suffering from malnutrition. Milk analyses revealed that the amount of fats, casein, milk-sugar, and salts was diminished while albumin increased as a result of insufficient nutrition.

McCollum and Davis[14] have shown that young rats maintained normal growth for periods of 70 to 120 days on a ration of purified food substances

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[2] Vernois, A.-G.-M., and Becquerel, A., *Ann. Hyg.*, 1853, xlix, 257; l, 43.

[3] Sebelien, J., *Z. physiol. Chem.*, 1885, ix, 445.

[4] Halliburton, W. D., *J. Physiol.*, 1890, xi, 448.

[5] Sebelien, J., *J. Physiol.*, 1891, xii, 95.

[6] Stoklasa, J., *Z. physiol. Chem.*, 1897, xxiii, 343.

[7] Filia, A., *Riv. clin. pediat.*, 1914, xii, 339.

[8] Schöndorff, B., *Arch. ges. Physiol.*, 1900, lxxxi, 42.

[9] Sherman, H. C., Berg, W. N., Cohen, L. J., and Whitman, W. G., *J. Biol. Chem.*, 1907, iii, 171.

[10] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1918, xxxiii, 7.

[11] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication No. 156*, 1911, pts. 1 and 2.

[12] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.

[13] Decaisne, E., *Gez. méd.*, 1871, xxvi, 317.

[14] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167.

consisting of casein, carbohydrate, and salt mixtures or the same ration in which a part of the carbohydrates was replaced by lard. Upon these rations females did not become pregnant; however, upon the addition of 1 gm. of ether extract of egg or butter, not only was growth resumed but they also became pregnant and gave birth to young. Some of the young animals were eaten by their mothers; others were reared but were very much undersized. The cause of the subnormal growth of young was due to the insufficient milk production of the mothers.

Osborne and Mendel,[15] working with young white rats, found that the milk food, which consisted of milk powder, 60 per cent, starch, 12 per cent, and lard, 28 per cent, was an adequate diet and animals not only had grown from infancy to full maturity, but also gave birth to young which in turn thrived upon the same diet.

Hart and Humphrey,[16] using two grades of Holsteins, showed that the nitrogen of alfalfa hay is quite efficient for milk protein building. From the fact that alfalfa hay contains a relatively small amount of acid amide nitrogen and a much greater amount of amino-acid nitrogen, they drew the conclusion that the real nutritive nitrogen value of alfalfa hay lay in the amino-acid nitrogen. They made[17] a comparison of the relative efficiency of the ingestion of the proteins of milk, corn, and wheat grain on milk production; they found milk protein most effective and wheat grain least effective. During the negative nitrogen balance which followed corn or wheat protein ingestion they observed enhanced tissue autolysis. The production of milk remained the same both in volume and concentration for a short period at the expense of catabolized tissue.

McCollum, Simmonds, and Pitz[18] found that young rats were able to grow normally upon a ration containing wheat, casein, dextrin, butter fat, and salt mixture. Females gave birth to litters of young, but the mothers failed to rear young on this diet. The lack of suitable milk production by the mother was due to the shortage of the water-soluble accessory factor in the milk. These results are the experimental proof of their earlier statement that the unidentified fat-soluble and water-soluble accessory substances of the diet "pass into the milk only as they are present in the diet of the mother, and that milks may vary in their growth-promoting power when the diets of the lactating animals differ widely in their satisfactoriness for the growth of young."[19]

Daniels and Nichols,[20] working with the soy bean rations, came to the conclusion that very young rats required a greater amount of fat-soluble accessory factor than adults to pass the early growing period successfully.

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[15] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.

[16] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1914, xix, 127.

[17] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1915, xxi, 239.

[18] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916–17, xxviii, 211.

[19] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 33.

[20] Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.

Loeb and Northrop[21] have been able to show that the imago of the fruit fly (*Drosophila*) can live on "glucose-agar" alone, while the larvæ cannot grow on "glucose-agar" unless yeast is added. The larvæ needed an adequate diet for body building while the imago, the full grown organism, appeared to be in no need of such growth-inducing substances as are present in yeast.

Stepp[22] has pointed out that the substance in cow's milk, which in minute quantities suffices to induce normal growth of young mice when added to an inadequate diet, is not fat, cholesterin, lecithin, or salts.

Meigs and Marsh[23] obtained two unknown substances from cow's milk, one alcohol-soluble and the other ether-soluble, which are claimed to be important constituents of diets. The former substance contained 13.8 per cent of nitrogen and its ash showed the presence of phosphorus; while the latter contained no nitrogen, but contained a considerable amount of sulfur. The amount of these substances diminished with the progress of lactation.

Methods and Material Employed.

The albino rats used by us were raised in our own laboratory. They were fed with wheat bread soaked in whole milk, fresh carrots, and occasionally a small amount of fresh meat (beef). This normal diet was designated as Ration N.

The animals were from 90 to 300 days old. This includes the period of rapid growth and the attainment of maturity. Also the most successful reproductions occur in this period. Pregnant animals on a normal diet were placed in separate metal cages, having sawdust- and newspaper-sprinkled floors, as soon as their condition was discovered and were put on the special diets which were continued until the end of the experimental period. In choosing the pregnant females among normally fed animals, care was taken to select only those in good health. A large number of pregnant rats were fed with the normal diet at the same time that the experimental diets were being investigated.

The animals were weighed together upon the day of birth (less than 12 hours after birth) and every alternate day. The size of the litter as well as the physical condition and body weight of the mother at the birth of the young and during the experi-

[21] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.
[22] Stepp, W., *Z. Biol.*, 1911–12, lvii, 135.
[23] Meigs, E. B., and Marsh, H. L., *J. Biol. Chem.*, 1913–14, xvi, 147.

mental period were recorded. The sex of the new-born animals was determined by the method devised by Jackson.[24]

It is a well known fact that generally male albino rats weigh more than females throughout life. King[25] has found in 85 litters examined that the average weight at birth of the male albino rats is 4.54 gm., and that of the females is 4.27 gm.; while Jackson[24] reported 5.13 gm. for males and 4.89 gm. for females in 63 and 66 animals respectively. The different factors which might influence the body weight of the albino rats at birth are summarized by King as follows: (*a*) The age of the mother; (*b*) the physical condition of the mother; (*c*) the body-weight of the mother; (*d*) the size of the litter; (*e*) the position of the litter in the litter series; and (*f*) the length of the gestation period.[25]

King's interesting graphic comparison[26] shows that during the first 60 days the growth curve of female albino rats runs very closely to the growth curve of the males, but then the curves begin to separate rapidly; *i.e.*, males surpassing females in body weight.

We have not attempted to weigh young males and females separately during the first 30 days for two reasons: first, there is no object in the determination of the body weights at definite ages; second, we are interested only in seeing what difference there is in the body weight and general condition of the young when the mother's diet is changed to the experimental diet.

The relative effective value of foods was determined by the change in the body weights of young rats at seven selected ages; namely, at birth, and on the 5th, 10th, 15th, 20th, 25th, and 30th days, and comparing these weights with those of young whose mothers were fed on a normal diet.

The following rations were employed in this investigation and the methods of preparation for the individual food substances are discussed in detail.

[24] Jackson, C. M., *Biol. Bull.*, 1912, xxiii, 171.
[25] King, H. D., *Anat. Rec.*, 1915, ix, 213.
[26] King, H. D., *Anat. Rec.*, 1915, ix, 751.

Ration N.

Bread.
Carrots.
Milk.

Ration Y.

	per cent
Bananas	83.5
Casein	16.0
Yeast	0.5

Ration M.

	per cent
Bananas	83.5
Casein	16.00
Yeast	0.5
Milk	10.0 cc. each animal.

Ration P.

	per cent
Bananas	83.0
Casein	16.0
Yeast	0.5
Protein-free milk	0.5

Ration S.

	per cent
Bananas	83.22
Casein	16.00
Yeast	0.50
Salt mixture	0.28

Ration N.S.

	per cent
Bananas	83.22
Casein	16.00
Yeast	0.50
Natural salt mixture	0.28

Ration L.

	per cent
Bananas	83.00
Casein	16.00
Yeast	0.50
Lactose	0.50

Bananas.—In a recent article Hess and Unger[27] have shown that fresh young vegetables possess a much greater amount of antiscorbutic and growth-promoting substances than old vegetables.

[27] Hess, A. F., and Unger, L. J., *J. Biol. Chem.*, 1919, xxxviii, 293.

Abderhalden and Lampé[28] and Funk[29] showed that cooked polished rice took much longer to produce polyneuritis in pigeons than uncooked polished rice. They suggested that the beneficial action of the cooked rice was due partly to the intake of relatively smaller amounts of carbohydrate by the birds.

In a preliminary communication,[1] we have reported that a banana diet maintained the life of young albino rats much longer than when fed on carrots as the sole food. We believed that the difference in the nutrition of young animals when fed upon these closely allied forms of foods is due partly to a difference in the degree of digestibility. Another example of how easily digestible foods influence the maintenance of animal life is seen in the fact that rats can live very much longer on the cooked white potato (Irish) than on uncooked potato. Detailed investigation on the nutritive value of potato will be given in a later paper. Throughout the experiments we used only the edible portion of well ripened, golden yellow bananas.

Casein.—McCollum and Davis have purified casein without any application of high temperature since they learned that the prolonged heating even at temperatures of 90–100°C. causes deterioration of the nutritive properties of milk. The growth curves showed the absence of unknown growth-promoting substances in their purified casein.[30] They have observed that heating casein in a moist condition for 1 hour in an autoclave at 15 pounds pressure destroys its biological value as a complete protein.[31] This particular point is true when a ration contained 5 per cent casein;[32] but when a ration contained 10 per cent casein,[33] the difference of the nutritive value in the heated and unheated casein is not clearly shown.

Funk and Macallum,[34] in order to free commercial casein from the traces of unknown accessory substances, extracted it by

[28] Abderhalden, E., and Lampé, A. E., *Z. ges. exp. Med.*, 1913, i, 296.

[29] Funk, C., *Z. physiol. Chem.*, 1914, lxxxix, 373.

[30] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 231.

[31] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 247.

[32] McCollum and Davis, [31] Chart 5.

[33] McCollum and Davis,[31] Chart 6.

[34] Funk, C., and Macallum, A. B., *Z. physiol. Chem.*, 1914, xcii, 13; *J. Biol. Chem.*, 1916, xxvii, 51.

refluxing for 6 hours with boiling 95 per cent alcohol. They found that the process did not alter the nutritive properties of the protein.

Drummond[35] has extracted the dried caseinogen with two changes of alcohol for 6 hours at 60°C., and then with ether for 6 hours. This method of purification did not make it unsuitable for the rats. However, he has observed in two cases that when the caseinogen was extracted with hot alcohol in a slightly moist condition, there was some chemical change and it lost its protein value.

The casein we employed was prepared from commercial, washed casein by boiling for 2 hours with 95 per cent ethyl alcohol. It was filtered after standing over night at room temperature and the casein washed well with fresh alcohol and then allowed to dry in the air. A former experiment[36] showed clearly that our purified casein was free from unidentified accessory factors, it was not toxic, and it possessed full biological value as a complete protein.

Yeast.—The shortage of water-soluble accessory substance in bananas was supplied from yeast.[37, 38] Fresh yeast[39] was filtered, pressed, dried in the air at room temperature, and was then well ground. During drying, mold has generally grown on the surface of the yeast.

Protein-Free Milk.—The fact that young white rats have failed to grow upon the isolated food substances, but rapid recovery of health and growth have followed when 28 per cent of protein-free milk has replaced the inorganic elements and a part of the carbohydrate in food, led Osborne and Mendel to conclude that their natural protein-free milk contains unidentified water-soluble accessory substances.[40] Later Osborne and Mendel[38]

[35] Drummond, J. C., *Biochem. J.*, 1916, x, 89.

[36] Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1919, xxxix, 421, Experiment 1.

[37] Funk, C., and Macallum, A. B., *J. Biol. Chem.*, 1915, xxiii, 413.

[38] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 149.

[39] The yeast was obtained from the Lion Brewery, New York City.

[40] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication No. 156*, 1911, pt. 2; *Z. physiol. Chem.*, 1912, lxxx, 356; *J. Biol. Chem.*, 1912, xii, 473; 1913, xv, 311; 1915, xx, 351; 1916, xxvi, 1.

have shown that the protein-free milk appeared to contain a new unidentified accessory substance which is not present in yeast.

We have prepared natural protein-free milk from fresh skimmed milk[41] according to the procedure used by Osborne and Mendel.[42] Different chemical analyses show that the contents of our preparations are nearly the same as those found by Osborne and Mendel.

Salt Mixtures.—The importance of the individual inorganic salts in the rôle of nutrition has been clearly shown and discussed.[42, 43] Our artificial salt mixture was prepared according to Osborne and Mendel.[44] The natural salt mixture was prepared by igniting our protein-free milk until entirely free from carbon. The inorganic residue gave 14.3 per cent of the original material.

Lactose.—Hopkins and Neville,[45] Sweet, Corson-White, and Saxon,[46] McCollum and Davis,[47] and Drummond[35] have clearly shown that the lactose, prepared from milk, may contain traces of impurities which act as a growth-promoting substance, and such substances can be removed by means of purification with 95 per cent alcohol.

We have purified lactose (Merck) by means of recrystallization from ethyl alcohol. The crystalline lactose was dried in a vacuum desiccator over sulfuric acid.

[41] The milk was obtained from the Walker-Gordon Laboratory Company, New York City.

[42] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication No. 156*, 1911, pt. 2.

[43] Röhmann, F., *Allg. med. Central.-Z.*, 1908, lxxvii, 129. McCollum, E. V., *Am. J. Physiol.*, 1909–10, xxv, 120. Evvard, J. M., Dox, A. W., and Guernsey, S. C., *Am. J. Physiol.*, 1914, xxxiv, 312. Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373. Hogan, A. G., *J. Biol. Chem.*, 1916, xxvii, 193. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 131. Steenbock, H., Kent, H. E., and Gross, E. G., *J. Biol. Chem.*, 1918, xxxv, 61. Sherman, H. C., Chemistry of food and nutrition, New York, 2nd edition, 1918, 234.

[44] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 317, Salt Mixture IV, minus the lactose.

[45] Hopkins, F. G., and Neville, A., *Biochem. J.*, 1913, vii, 97.

[46] Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *J. Biol. Chem.*, 1915, xxi, 309.

[47] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.

Experimental Results.

Our experimental results are compiled in Tables I to VII.

From the experiments we obtained the following facts:

(1) The comparative number of young born per litter from mothers fed with different rations is summarized in Table VIII. (2) The average number of days when the young from mothers fed on the different diets opened their eyes was noted and found to be 15.6 for Ration N, 16.0 for Ration Y, 16.0 for Ration M, 15.6 for Ration P, and 15.9 for Ration L. (3) The total per cents of young rats which were born under the following diets and which have thrived during the experimental periods of 30 days were found to be with the normal diet, 71; with Ration Y, 20; with Ration M, 87; with Ration P, 71; and with Ration L, 26. (4) The addition of 0.5 per cent of protein-free milk to the mother's diet, which consisted of bananas, 83.0, casein, 16.0, and yeast, 0.5 per cent, not only maintained her body weight throughout the lactation period, but also gave excellent milk supply both in quantity and quality (Table IV). (5) The amount of the food, Ration P, eaten by the mother during the lactation period increased as lactation progressed. (6) The addition of 0.28 per cent of salt mixture, either artificial or natural, had no beneficial action upon the banana-casein-yeast diet (Tables V and VI). (7) The addition of 0.5 per cent of purified lactose to the banana-casein-yeast diet increased slightly its nutritive value. A small number of young were reared by the mothers on this diet, but their body weights were very much below normal (Table VII). (8) Table IX shows more clearly the relative effective value of foods. The weights are the average of the rats which survived during the experimental periods. These figures are compiled from Tables I to VII.

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TABLE I.

Ration N.—Bread, Carrots, Milk.

Mother.		No. of litter.		Average body weight of males and females together.							Remarks.
No.	Body weight	Males.	Females.	Birth.	5th day.	10th day.	15th day.	20th day.	25th day.	30th day.	
	gm.			gm.	gm.	gm.	gm.	gm.	gm.	gm.	
60	147	4	6	3.81	5.66	7.45	9.83	13.30	17.27	22.08	1 ♀ died on the 17th; 1 ♂ on the 18th; 1 ♀ on the 21st day; rest grew; somewhat undersized.
60	160	5	5	3.90	*						1 ♂ died shortly after birth; rest died on the 2nd day.
282	130	4	5	4.93	7.23	10.72	13.93	17.72	21.50	24.18	Animals grew well; general condition excellent.
283	178	4	3	5.21	9.70	13.59	†				1 ♀ died shortly after birth; rest killed and eaten on the 12th day.
294	204	2	3	5.62	10.17	16.76	24.15	32.92	41.74	47.04	Animals grew well; general condition excellent.
219	138	7	1	5.00	7.41	10.12	11.55	13.26	17.52	21.80	" " somewhat undersized.
104	178	7	1	4.85	8.05	11.27	14.79	18.18	21.96	24.05	" " well; general condition excellent.
374	198	9	3	5.03	8.04	10.91	13.26	†			Killed and eaten on the 17th day.
376	144	4	5	4.35	7.31	12.26	16.33	20.29	25.01	31.77	1 ♂ and 1 ♀ eaten on the 2nd day; rest grew well; general condition excellent.
377	163	5	5	4.74	7.26	11.12	14.94	19.91	24.45	30.89	Animals grew well; general condition excellent.
431	153	5	3	5.09	7.74	10.51	13.63	18.05	22.90	28.30	" " " " " "
445	106	3	1	5.15	8.39	12.92	18.25	25.40	32.90	41.00	" " " " " "
249	167	7	3	4.34	5.77	8.19	11.18	14.59	17.99	22.09	" " somewhat undersized.
481	225	4	2	5.98	11.04	17.90	25.20	32.98	43.34	51.84	" " well; general condition excellent.

* Died.

† Animals were killed and eaten by mother.

TABLE II.

Ration Y.—Bananas 83.5, Casein 16.0, Yeast 0.5 Per Cent.

Mother.		No. of litter.		Average body weight of males and females together.							Remarks.
No.	Body weight.	Males.	Females.	Birth.	5th day.	10th day.	15th day.	20th day.	25th day.	30th day.	
	gm.			gm.	gm.	gm.	gm.	gm.	gm.	gm.	
65	126	3	2	4.33	*						2 ♂ died on the 1st day; 1 ♂ and 1 ♀ on the 3rd day: 1 ♀ on the 4th day.
77	117	2	0	5.01	*						All died on the 1st day.
65	117	4	5	2.93	*						2 ♀ died on the 1st day; rest died on the 2nd day.
193†	128	3	3	4.45	6.00	6.02	*				2 ♀ and 1 ♂ died on the 9th; on the 11th 1 ♂; on the 12th 1 ♂ and 1 ♀.
219†	135	5	7	3.73	6.13	8.43	10.48	11.33	12.62	16.53	1 ♂ died on the 26th day, and 1 ♂ eaten; rest poor and development retarded.
220†	103	5	4	4.16	5.13	7.28	9.40	11.03	‡		3 ♂ and 2 ♀ died on the 21st day, and 1 ♂ and 2 ♀ eaten; on the 22nd day 1 ♂ died.
62†	125	3	4	4.39	4.00	‡					1 ♂ died on the 2nd day; 1 ♂ died and 1 ♀ eaten on the 4th; on the 6th 3 ♀ died and 1 ♂ eaten.

* Died.

† Animals were on normal diet when they became pregnant.

‡ Animals were killed and eaten by mother.

TABLE III.

Ration M.—Bananas 83.5, Casein 16.0, Yeast 0.5 Per Cent and Milk 10 Cc. for Each Animal.

Mother.		No. of litter.		Average body weight of males and females together.							Remarks.
No.	Body weight.	Males.	Females.	Birth.	5th day.	10th day.	15th day.	20th day.	25th day.	30th day.	
	gm.			gm.	gm.	gm.	gm.	gm.	gm.	gm.	
49*	132	5	4	3.98	7.00	8.86	13.38	17.18	21.78	26.13	2 ♂ and 2 ♀ died on the 2nd day; rest grew well; general condition excellent.
91*	124	4	5	4.02	6.72	9.57	11.63	12.82	15.80	19.15	Animals grew; somewhat undersized.
283†	151	5	2	5.05	7.44	13.14	17.43	22.00	26.95	32.17	" " well; general condition excellent.
219†	144	3	3	4.27	6.60	9.86	12.27	17.91	22.09	28.14	" " " " " "

* Animals were on Ration Y when they became pregnant.

† Animals were on normal diet when they became pregnant.

TABLE IV.

Ration P.—Bananas 83.0, Casein 16.0, Yeast 0.5, Protein-Free Milk 0.5 Per Cent.

Mother.		No. of litter.		Average body weight of males and females together.							Remarks.
No.	Body weight.	Males.	Females.	Birth.	5th day.	10th day.	15th day.	20th day.	25th day.	30th day.	
	gm.			*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
65*	123	3	2	4.80	6.64	9.94	13.67	16.97	23.31	32.89	Animals grew well; general condition excellent.
210	124	1	5	4.35	7.35	11.55	15.50	19.72	26.96	32.91	" " " " " "
222*	130	4	3	4.22	†						All died on the 1st day.
49*	139	4	3	4.29	6.77	11.52	15.26	18.54	27.12	36.17	Animals grew well; general condition excellent.
104*	183	4	7	5.15	8.35	11.92	14.32	15.78	20.31	24.20	General condition poor and development retarded.
196*	143	2	3	4.91	10.67	16.14	20.64	27.16	38.11	45.51	Animals grew well; general condition excellent.
248*	152	4	3	5.01	8.83	12.46	‡				1 ♂ died on the 2nd day; during 11th day all eaten.

* Animals were on normal diet when they became pregnant.

† Died.

‡ Animals were killed and eaten by mother.

TABLE V.

Ration S.—Bananas 83.22, Casein 16.00, Yeast 0.50, Salt Mixture 0.28 Per Cent.

Mother.		No. of litter.		Average body weight of males and females together.							Remarks.
No.	Body weight.	Males.	Females.	Birth.	5th day.	10th day.	15th day.	20th day.	25th day.	30th day.	
	gm.			gm.	gm.	gm.	gm.	gm.	gm.	gm.	
214*	117	4	3	4.06	†						1 ♂ and 1 ♀ died shortly after birth; rest died on the 2nd day.
217*	108	4	4	3.53	†						3 ♂ and 2 ♀ died on the 1st day; rest died on the 2nd day.
282*	142	2	3	4.88	5.24	‡					All eaten on the 6th day.

* Animals were on normal diet when they became pregnant.
† Died.
‡ Animals were killed and eaten by mother.

TABLE VI.

Ration N.S.—Bananas 83.22, Casein 16.00, Yeast 0.50, Natural Salt Mixture 0.28 Per Cent.

Mother.		No. of litter.		Average body weight of males and females together.							Remarks.
No.	Body weight.	Male.	Female.	Birth.	5th day.	10th day.	15th day.	20th day.	25th day.	30th day.	
	gm.			gm.	gm.	gm.	gm.	gm.	gm.	gm.	
376*	198	5	3	4.07	5.28	†					1 ♂ and 1 ♀ died on the 3rd; on the 4th 2 ♂ and 1 ♀; and on the 6th the rest died.
374*	162	2	4	4.32	‡						Killed and eaten on the 2nd day.

* Animals were on normal diet when they became pregnant.
† Died.
‡ Animals were killed and eaten by mother.

TABLE VII.

Ration L.—Bananas 83.0, Casein 16.0, Yeast 0.5, Lactose 0.5 Per Cent.

Mother.		No. of litter.		Average body weight of males and females together.							Remarks.
No.	Body weight.	Male.	Female.	Birth.	5th day.	10th day.	15th day.	20th day.	25th day.	30th day.	
	gm.			*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
60	157	5	3	4.22	†						Eaten on the 2nd day.
234	111	2	3	4.64	7.00	10.04	13.71	16.25	19.24	20.52	Animals grew; somewhat undersized.
217	121	2	4	5.14	4.70	‡					1 ♀ died on the 5th; on the 6th day 2 ♂ and 2 ♀ died and 1 ♀ eaten.
219	142	?		†							Killed and eaten by mother.
280	131	6	2	3.87	4.62	†					2 ♂ died on the 2nd day; on the 3rd 2 ♂ and 1 ♀ eaten; on the 6th 1 ♂ and 1 ♀ eaten; rest died on the 7th day.
220	134	5	2	4.13	5.76	7.43	8.89	9.97	11.86	13.63	General condition poor; development retarded.
217	142	3	3	5.00	6.00	‡					All young died during the 8th day.
378	127	5	2	5.32	7.33	8.58	7.58	8.35	‡		2 ♂ and 1 ♀ died on the 15th day; on the 16th 1 ♀; on the 20th day 3 ♂.
385	151	2	4	5.02	7.03	10.50	14.44	19.18	23.62	28.09	Animals grew well; general condition excellent.
411	119	5	4	4.21	7.04	9.79	16.68	19.58	23.13	27.90	1 ♀ eaten on the 2nd day; on the 3rd 1 ♂ eaten; on the 9th 1 ♀; on the 11th day 1 ♂ died; rest grew well; general condition excellent.
341	113	3	3	3.93	5.37	7.03	6.75	6.23	‡		1 ♂ died shortly after birth; on the 2nd 1 ♀; 1 ♂ on the 17th; 1 ♀ on the 18th; rest died on the 20th day.
374	155	2	3	4.02	†						Mother ate all on the 2nd day.

473	152	2	6	4.78	6.90	7.25	8.39	8.65	9.79	10.60	1 ♂ eaten on the 2nd day; on the 13th 1 ♀ died; on the 24th 2 ♀ died; on the 28th 1 ♀; on the 29th 2 ♀ eaten; rest, general condition poor; development retarded.
376	190	5	3	4.81	7.66	12.18	15.26	17.48	19.64	22.66	2 ♂ eaten on the 2nd day; on the 6th day 1 ♂ eaten; rest grew, somewhat undersized.
249	130	6	4	3.30	†						5 eaten on the 1st day; on the 2nd 1 ♂ and 1 ♀; on the 3rd 1 ♂ and 1 ♀; on the 4th 1 ♂.
276	115	6	0	4.03	4.13	‡					All young died on 7th day.
341	118	4	3	4.57	4.38	‡					" " " " 8th "
385	116	3	2	4.59	7.12	11.82	16.65	22.16	26.24	29.82	1 ♂ eaten shortly after birth; on 2nd day 1 ♂ and 2 ♀ eaten; rest grew well; general condition excellent.

* All animals were on normal diet when they became pregnant.
† Animals were killed and eaten by mother.
‡ Died.

TABLE VIII.

Mother's ration.	No. of litters examined.	Average no. of young per litter.
N.	14	8.3
Y.	7	7.1
M.	4	7.8
P.	7	6.9
S.	3	6.7
N.S.	2	7.0
L.	18	6.5

TABLE IX.

Day of determination.	Weight of rats on experimental diets.						
	Ration N	Ration Y.	Ration M.	Ration P.	Ration S.	Ration N. S.	Ration L.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Birth.	4.86	4.14	4.33	4.68	4.16	4.20	4.45
5th.	7.98	5.32	6.94	8.10	5.24	5.28	6.07
10th.	11.82	7.24	10.36	12.26	*	*	9.40
15th.	15.59	9.94	13.68	15.88			12.03
20th.	20.60	11.18	17.48	19.63			14.21
25th.	26.05	12.62	21.66	27.16			19.07
30th.	31.37	16.53	26.40	34.34			21.89

* Died.

DISCUSSION.

Our results show that whole milk, or protein-free milk is effective in making a banana-casein-yeast diet complete for milk production.

According to Osborne and Mendel,[11] their protein-free milk contains 0.48 per cent of non-protein nitrogen, and 0.28 per cent of protein nitrogen. They have stated that the amount of milk protein in the protein-free milk was not the cause of inducing the growth of the retarded animals. On the other hand, McCollum and Davis[48] argue that the nitrogen of the protein-free milk is equivalent to milk protein nitrogen as a nutrient for young rats. Osborne and Mendel[38] answer the criticism of McCollum

[48] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xx, 641.

and Davis by showing very remarkably different chemical properties possessed by the protein-free milk and yeast. They say:

"On a ration of purified casein, 'artificial protein-free milk,' starch, lard, butter fat, and 1.5 per cent of dried yeast, rats of both sexes have grown from about 50 gm. body weight to maturity, and have even produced young. . . . . Adult rats have been maintained for more than 300 days. For some as yet unknown reason the majority of the rats grew normally when the protein used was casein, whereas they have usually failed when it was edestin, and almost invariably when lactalbumin, cotton seed globulin, cotton seed proteins, or squash seed globulin was fed. This result surprised us because all of these proteins[49] had earlier led to normal growth when used in rations containing natural 'protein-free milk.' "

From these experiments, Osborne and Mendel make the suggestion that the unknown nitrogenous constituents in the protein-free milk may supply the unrecognized deficient substances in these proteins. Assuming Osborne and Mendel's figures to be correct, we added only 0.0088 per cent of milk protein from the protein-free milk. We believe this amount does not exercise any influential effect upon the nutritive efficiency of the dietary.

Interesting investigation on the nature of lactalbumin as a complete protein has been made by Emmett and Luros.[50] They concluded, from the fact that lactose supplemented lactalbumin, that the former either acted as a buffer to overcome the toxicity present in the diet, or it adsorbed a new water-soluble growth-promoting substance.

In a recent paper, Kennedy[51] has reported that protein-free milk contains "either unprecipitated protein, or peptids of considerable size."

CONCLUSIONS.

1. A diet consisting of bananas, 83.0 per cent, casein, 16.0 per cent, yeast, 0.5 per cent, and protein-free milk, 0.5 per cent is an adequate diet for the growth, maintenance, reproduction, and perfect milk production of the albino rats.

[49] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1912, xii, 473; 1915, xx, 351; 1916, xxvi, 1.

[50] Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, 1919, xxxviii, 147.

[51] Kennedy, C., *J. Am. Chem. Soc.*, 1919, xli, 388.

2. Protein-free milk contains a substance which is needed for suitable milk production by the mother.

3. This substance appears not to be associated with purified milk-sugar or inorganic constituents of milk.

4. This peculiarity possessed by the protein-free milk indicates that it contains a new accessory substance which is lacking in yeast.

5. Our experiments suggest that a combination of bananas and milk, in proper proportion, constitutes a complete food.

FURTHER CONTRIBUTIONS TO THE PHYSIOLOGY OF PHOSPHORUS AND CALCIUM METABOLISM OF DAIRY COWS.*

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(Received for publication, September 24, 1919.)

Recent work has made it seem probable that the amount of calcium and phosphorus contained in the rations of dairy cows is a matter of great practical importance. In a comprehensive series of experiments on liberally milking cows, Forbes and coworkers[1] have found that the calcium balances were always negative and the phosphorus balances usually so; and this in spite of the fact that the rations were liberal and, in many cases, contained more calcium and phosphorus than is ordinarily fed even to high producing cows.

In view of these and other results with the same general tendency, it seems desirable that calcium and phosphorus metabolism in dairy cows be intensively studied; and we have recently carried out a series of experiments on cows in which the calcium, phosphorus, and nitrogen balances were followed.

Objects of the Experiments.

Our experiments were planned to throw light on a number of points. It has been the custom at the Government Farm at Beltsville, Md., as it probably is on most dairy farms, to feed cows according to their milk yield. As the milk yield decreases

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[1] Forbes, E. B., Beegle, F. M., Fritz, C. M., Morgan, L. E., and Rhue, S. N., *Ohio Agric. Exp. Station, Bull. 295*, 1916; *ibid., Bull. 308*, 1917. Forbes, E. B., Halverson, J. O., and Morgan, L. E., *ibid., Bull. 330*, 1918.

with the progress of lactation, the feed—particularly the grain—is reduced, and reaches its lowest point when the cow becomes altogether dry, generally only a few weeks before the next calf is born. The routine ration fed to the dry cows at Beltsville is shown in Table III, as the ration fed to Cow 51 from February 17 to March 10, 1919. It contains sufficient protein, fat, and carbohydrate to provide liberally for the maintenance of the cow and the growth of the unborn calf, as calculated from the figures given in the standard text-books.

In most dairy rations, the grain contains a large proportion of the phosphorus, and the ration in question is no exception to this rule (Table V). As it is the grain which is chiefly reduced in the rations of the dry cows, it becomes an interesting question whether these cows receive sufficient phosphorus to provide for the optimum milk yield in the next lactation.

We have recently reported experiments in which the concentration of phosphorus in the blood plasma of dry, pregnant cows has been followed.[2] Though the cows were fed a much higher grain ration than the one in question, there was nevertheless a decided tendency for the concentration of phosphorus in the blood plasma to fall off toward the end of pregnancy. In other unpublished experiments, we have found that this tendency is more marked when the grain ration is lower.

We are engaged at present in carrying out experiments to determine whether the ration described above can be improved from the practical standpoint by adding sodium phosphate to it, and by feeding it in a manner which we judged would facilitate the absorption of phosphorus. There is considerable evidence to show that the absorption of phosphorus from the intestinal tract may be hindered by the simultaneous presence of calcium compounds.[3,4] We have, therefore, attempted to separate the calcium

[2] Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 1. The experiments in question are referred to on pp. 45 and 46.

[3] Hammarsten, O., A text-book of physiological chemistry, translated by Mandel, J. A., New York and London, 7th edition, 1915, 762. Forbes, E. B., with collaborators, *Ohio Agric. Exp. Station, Techn. Bull. 6*, 1915, 66.

[4] Bertram, J., *Z. Biol.*, 1878, xiv, 335.

and phosphorus compounds in the intestinal tract by feeding the grain and hay of the rations on alternate days. This method of feeding is shown in Table III as the ration fed to Cow 63 from February 17 to March 10.

The milk yield of cows fed, for several weeks before calving, in this manner has been compared with that of control animals fed like Cow 51, February 17 to March 10 (Table III). So far, about a dozen experiments and controls have been carried through altogether, and the results indicate that the cows on the experimental feeding gain weight and improve in condition at least as satisfactorily as the controls and that their milk yield in the next succeeding lactation is decidedly larger.

We do not consider the results sufficiently numerous as yet to be final, and we are carrying out further experiments of the same sort. But, in the meantime, we wished to study the effects of the experimental feeding on calcium and phosphorus assimilation, and the experiments to be reported in this article have been planned with that end in view.

This made it possible to study the effects of feeding phosphate on the urinary and fecal excretion of phosphorus. We have previously shown that feeding phosphate tends to increase the concentration of phosphorus in the blood.[5] It seemed worth while, therefore, to take a certain number of blood samples during the course of the balance experiments and to study the triple relationship between phosphorus in the rations, in the blood, and in the urine.

General Plan of Experiments.

Two healthy, grade Holstein cows were selected, each of which had been dry for about a month. Each had been fed for some time previous to the beginning of the experiment on the same ration that she was to have during the first part of the experiment. Both were in the last third of pregnancy. One, No. 51, was due to calve on April 17, 1919, 59 days after the beginning of the experiment, the other, No. 63, on May 4, 1919, 76 days after the beginning of the experiment. Their weights are given in Table I.

[5] Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 45.

On February 17, 1919, the cows were brought into the stalls in which the experiment was to be carried out. These stalls, in the basement of the laboratory building, were built of wood. Their construction was in general similar to that to which the cows were accustomed. They were provided with mangers so constructed as to prevent effectively the scattering of the rations, and with beds made of several layers of sacking. The cows were held in stanchions like those to which they were accustomed. The temperature in the experiment room ranged from 10–18°C.

The urine and feces were collected in essentially the same manner as in Forbes'[1] experiments. Three men were employed, each of whom remained behind the cows for 8 hours out of the 24, and were provided with shovels and large, galvanized iron dippers in which to catch the feces and urine, respectively, before they reached the ground. In addition, a long, galvanized iron pan was placed behind each cow to catch any of the excreta that might accidentally fall. The feces caught in the shovels were transferred to covered, galvanized iron garbage cans, and the urine caught in the dippers was transferred to glass carboys. The excreta which fell accidentally were placed in separate receptacles and weighed. The total amount, however, throughout the course of the experiments was so small that no conceivable departure of its composition from the average could have had any perceptible effect on the results. It was therefore simply brought into the calculation as a part of the daily amount of excreta voided, but was not separately analyzed.

The experimental day began and ended at 10 a.m. At this time the urine and feces collected in the previous 24 hours were weighed and aliquot samples of each were set aside. As an aliquot from the feces we took $\frac{1}{5}\frac{1}{0}$ of the total amount voided in the 24 hours and added a small amount of chloroform as a preservative. From the urine we took two aliquots in each case, one for the nitrogen determination, and the other for the calcium and phosphorus determinations. The first of these amounted to 20 cc. per pound of urine voided, and was preserved with a small amount of chloroform. The second amounted to 100 cc. per pound of urine voided. To this quantity enough strong HCl was added to make the resulting mixture distinctly acid to litmus after all the CO_2 had been driven off. The amount of HCl added was measured and recorded, and a small amount of chloroform was added.

The experimental period lasted in each case for 4 days. The aliquots obtained during this period were thoroughly mixed, and in this way representative samples were obtained for analysis. The results, however, are given as the daily average for each of the 4 day periods.

On February 17, 1919, a blood sample was obtained from each cow at about 8 a.m.; the cows were brought into the experimental stalls about 9 a.m., and their urine was collected for the next 18 hours. From February 18 until March 2, 1919, inclusive, the urine and feces were not collected, but otherwise the cows were kept under the same routine as during the collection periods. The rations given to each are shown in Table III.

The balance experiment began on March 3. About 8 a.m. on this date a second blood sample was obtained from each cow, and at 10 a.m. the collection of the urine and feces was begun. This was continued until 10 a.m. on March 11, the intervening 8 days being divided into two 4 day collection periods as described above. On the morning of March 11, a third blood sample was obtained from each cow, and the rations were reversed as shown in Table III. The period from March 11 to 20, inclusive, served as an intermediate period to accustom the cows to the new rations. The excreta were not collected.

On the morning of March 21, it was evident that No. 51 was going to abort, and she had to be discarded as a subject for the experiment. The abortion took place that evening. The calf was born dead, but it weighed about 30 kilos and was well formed for a calf born 4 weeks too early. A few weeks later No. 51 was tested for contagious abortion and reacted positively.

It was decided to carry the experiment on with No. 63 as originally planned, and to substitute for No. 51 another cow, No. 66, which had for 88 days previously been receiving the same alternated rations that were supplied to No. 51 from March 11 to 20. This cow was a grade Guernsey, and was neither pregnant nor milking, but it was thought that some further data could be obtained from her in regard to the effects of phosphate feeding on the concentration of phosphorus in the blood and the elimination of phosphorus and calcium in the urine.

At 7.30 on the morning of March 21, therefore, blood samples were obtained from Nos. 63 and 66 and at 10 a.m. the collection

of urine and feces was started. It was continued as before for two 4 day periods. At the end of the second one, blood samples were again taken and the experiment was concluded.

No. 63 gave birth to a normal calf, which weighed 31.4 kilos, on the morning of April 25, 1919.

The cows were, of course, closely watched for any abnormalities of appetite or general behavior. No. 51 showed no outward sign of disturbance by the experimental routine at any time, except that she drank less water during the collection periods than during the intermediate period (Table II).

No. 63, on the other hand, gave signs of being disturbed by various parts of the procedure. During the preliminary period (February 17 to March 2), she refused a part of her ration, although she had eaten it well for the entire previous 2 weeks in the general barn; her silage had to be reduced. She consumed the reduced ration for a day or two, but began to refuse some of it again as soon as the collection of urine and feces began. During the first 60 hours of the first collection period, she did not lie down in her stall, probably on account of abrasions which had developed on her hips and shoulders, and toward the end of this 60 hours she became obviously extremely nervous and uncomfortable. The difficulty was overcome by providing her with sacks filled with straw for a bed.[6] Like No. 51, she drank less water in the collection periods than in the intermediate period. She also consistently refused some of her hay and silage in the collection periods in spite of the fact that her ration had been cut down. During the intermediate period, March 11 to 20, she refused only very small portions of her feed (Table IV).

No. 66, March 21 to 28, suffered, of course, from the double disturbance of new surroundings and collection of urine and feces. Her appetite was not much affected. She ate her rations, except for the stems of the hay and the cobs in the silage, but she drank very little water, and was obviously nervous under the experimental routine. She would start when anyone went near her or touched her, and would often begin the act of urination, and then discontinue it when the attendant approached her.

[6] All cows were provided with beds of this sort from the beginning of the intermediate period, Mar. 11 to 20.

Analytical Methods.

In undertaking our analyses of the materials fed to the cattle, we realized keenly the difficulties which might lead us into error. The errors to be expected may be summed up under two heads; those due to the fact that all the feeding materials contain a considerable and more or less inconstant percentage of water, and those due to the difficulty of obtaining representative samples for analysis—particularly from such materials as corn silage and alfalfa hay. Our preliminary experience showed us that these difficulties are very real.

They are to be avoided by grinding large samples, by thorough mixing of the ground material, and by making sufficiently numerous moisture determinations. The process of grinding often produces very considerable changes in water content, which must be adequately controlled.

We evolved a procedure which gave satisfactory results, but we do not think it necessary to give it in detail. Its detailed description would occupy a great deal of space, and the precautions are such as would occur to anyone seriously interested in the subject. We prepared enough hay and grain at the beginning to last through the experiment; but with the silage we found it advantageous to take out aliquot samples for analysis from each feed. The aliquots for each collection period were thoroughly mixed and analyzed separately from those of the other periods, and in our tables, therefore, the composition of the silage fed is given for each period separately. The composition of the various feeds is given in Table V; and the composition of the feed refused, in Table VI. In a footnote to Table IV are given details concerning the manner in which the feed refused was handled.

The analytical methods used, were, in general, those described in our previous article.[5] Some modifications have, however, been introduced as indicated below.

For calcium determinations we ashed some of our samples of feces and some of our samples of hay in platinum crucibles over a free flame or in an electric furnace, instead of with nitric and sulfuric acids. We found it most convenient to determine calcium in urine directly without ashing, as suggested by McCrudden.[7]

[7] McCrudden, F. H., *J. Biol. Chem.*, 1911–12, x, 199.

In some calcium determinations, also, in material ashed with nitric and sulfuric acids, we omitted the preliminary precipitation of the calcium as sulfate, our new procedure being as follows:

The acid ash is boiled with about 25 cc. of water as usual and then made up to approximately 75 cc. with water. One drop of 0.006 per cent phenolsulfonephthalein is added and 28 per cent NH_3 until the color changes through yellow to pink. The mixture is cooled and 10 per cent HCl is added drop by drop until the color changes back to clear yellow; 10 cc. of 0.5 N HCl and 10 cc. of 1.75 per cent $H_2C_2O_4$ are then added; the mixture is heated to boiling; 20 cc. of saturated $(NH_4)_2C_2O_4$ solution are added; and the mixture is kept gently boiling until the precipitate becomes coarse (15 to 30 minutes). It is then cooled to 50° or below, 10 cc. of 10 per cent $NaC_2H_3O_2$ are added, and the calcium in the oxalate precipitate is determined according to the procedure given in our earlier article.

With these exceptions our analyses were carried out exactly as described in our earlier article; and, in the cases where we departed from the old procedure, we carried out parallel analyses on the same samples of material and satisfied ourselves that the two methods gave the same results. We determined the moisture in our material by drying to constant weight at a temperature between 103 and 107°.

We had a good deal of difficulty with the urine. Cow's urine often contains so little calcium and phosphorus that large samples must be taken if accurate results are to be obtained. To evaporate these large samples and ash the residues over a flame is very tedious and troublesome, while, if the samples are ashed with acid mixture, much difficulty is encountered on account of the high content of hippuric acid which changes to benzoic acid during the ashing. After trying various procedures we found that it was most satisfactory to determine calcium without ashing as described above, and to determine phosphorus in samples which had been ashed with sulfuric and nitric acids. The aliquot samples of urine were immediately mixed with enough hydrochloric acid to drive off all the carbonate and render them distinctly acid to litmus, and this treatment brought about the precipitation of a large proportion of the hippuric acid. Calcium and phosphorus were determined after filtering this off. For the determination of nitrogen,

of course, it was necessary to set aside other aliquots not acidified and preserved only with a small amount of chloroform.

The blood samples were treated and analyzed as described in our previous article, except that in some cases a change was made in the procedure for calcium, as described above.

In regard to the size of the samples and the accuracy of the determinations in the case of the blood, the remarks given in our earlier article apply also to this investigation. The figures given in our tables for the composition of blood and plasma are the averages of duplicates which generally agreed with one another within 5 per cent.[8] The same remark applies also to our figures for the composition of the urine. For the feeds and feces we were able to take optimum samples, and the duplicates generally agreed more closely.

The results of our experiments are given in Tables I to XII at the end of the article.

Calcium and Phosphorus Metabolism in Balance Experiments.

Our results show a peculiar relation between calcium and phosphorus assimilation which seems to us to call for very careful consideration. In every period except that of March 3 to 6, in the case of No. 51, phosphorus was assimilated in larger absolute quantity than calcium. The phosphorus balances were positive throughout, while two out of the eight calcium balances were negative.

In six out of the eight periods, we were dealing with cows which were carrying calves, and at or beyond the 7th month of pregnancy. It is possible to form an approximate estimate of the rapidity with which calcium and phosphorus are assimilated by the embryo calf in the last 2 months of its intrauterine existence. A calf, according to Forbes and Keith,[9] contains about 1.65 per cent CaO and 1.53 per cent P_2O_5 in its body, and a new-born calf weighs

[8] The difference obtained by subtracting one figure from the other was 5 per cent or less of the larger.

[9] Forbes, E. B., and Keith, M. H., *Ohio Agric. Exp. Station, Techn. Bull. 5*, 1914, 106.

about 35 kilos. A new-born calf, therefore, would have about 413 gm. of calcium and 234 gm. of phosphorus in its body.

Forbes and Keith (page 110)[9] give the weights and the CaO and P_2O_5 contents of human embryos at various stages. As the human and bovine gestation periods are very nearly the same, the figures given may be taken as an approximate representation of the progress of things in the case of the calf. The human embryo at 7 months weighs about one-third of what it weighs at term, and its body contains about two-thirds the percentage of calcium and phosphorus that is contained in that of the new-born infant. The 7 months embryo, therefore, would contain about two-ninths of the absolute quantities of calcium and phosphorus contained in the new-born infant or calf. $\frac{2}{9}$ of 413 is approximately 92, and $\frac{2}{9}$ of 234 is 52. In the last 60 days of intrauterine life, therefore, the embryo calf must assimilate about 321 gm. of calcium or 5.3 gm. a day, and about 182 gm. of phosphorus, or 3.0 gm. a day. These figures are of course only rough approximations, but they are quite accurate enough for our purposes.

Reference to Table X shows that the daily phosphorus assimilation of Cows 51 and 63 was sometimes above and sometimes below 3.0. The average for the six periods in which these cows were studied is 3.33, which corresponds satisfactorily with the calculated figure.

The results with regard to calcium metabolism are strikingly different. The calcium assimilation of our pregnant cows was always well below 5.3, the nearest figure to this being 3.5 for No. 51, March 3 to 6. The average daily calcium assimilation for our pregnant cows was only 1.44 gm. This means that if the calves were growing at all as they should during the periods of the balance experiments their mothers must have been transferring considerable quantities of calcium from their own bones to those of their offspring.

One's first idea is that our rations must have been markedly deficient in calcium. We do not think that this was the case. We fed alfalfa hay, and the rations contained from 30 to 40 gm. of calcium daily. This quantity is generally considered liberal; and if our rations had been markedly deficient in calcium, the bad effects should have shown themselves in imperfectly formed calves and in reduced subsequent milk yield; because both cows were on

approximately the same rations they received during the collection periods for from 7 to 11 weeks before calving. None of these bad effects showed itself. It is true that No. 51 aborted, but there was much contagious abortion in the herd at the time, and she was shown to have the disease. The premature calf was large and well formed for a calf born nearly 4 weeks ahead of time, and had certainly died only a few hours before its birth. No. 63 gave birth to a fair sized and perfectly normal calf at about the right time. Both cows have given much more milk in the lactation periods succeeding the experiments than they had ever given before.

We think that the true explanation of our results on calcium and phosphorus assimilation is quite different from that which has been suggested; and, in order to bring out certain important aspects of the subject, we wish to call attention to the proportions of calcium and phosphorus contained in the bodies of certain mammals, and to the results which have been obtained in balance experiments on the same species of mammals in the past.

The ratio $\frac{Ca}{P}$ contained in the whole bodies of mammals is fairly constant, and somewhat higher in mature than in new-born animals. For mature cattle and sheep this ratio is about 1.9:1; for mature pigs about 1.6:1.[10] Growing cattle and sheep must therefore, on the average, assimilate at least 1.9 parts by weight of Ca for 1 part by weight of P; growing pigs must assimilate about 1.6 parts of Ca for 1 part of P. The figures obtained in balance experiments carried out in the past deviate largely from these ratios and almost universally in the same direction.

We have made a representative study of the balance experiments carried out in the last 60 years, selecting those which included work on the calcium and phosphorus balances of healthy growing animals fed on normal or nearly normal rations.[11, 12, 13] In these

[10] Forbes and Keith.[9] These ratios represent absolute weights, not equivalents.

[11] Lehmann, J., *Landw. Ver.-Stat.*, 1859, i, 68. von Gohren, T., *ibid.*, 1861, iii, 161. Soxhlet, Erste Bericht über Arbeiten der landwirtschaftlichen-chemischen Versuchs-Station in Wien, 1870–71, 101. Weiske, H., *J. Landw.*, 1873, xxi, 139. Hofmeister, V., *Landw. Ver.-Stat.*, 1873, xvi, 126. Köhler, A., Honcamp, F., Just, M., Volhard, J., Popp, M., and Zahn

twelve researches are included the results of 136 separate experiments. In all but eighteen of these the ratio $\frac{\text{Ca assimilation}}{\text{P assimilation}}$ is less than 1.9 for the cattle and sheep, and less than 1.6 for the pigs.

The unanimity with which balance experiments on growing animals show a deficiency in calcium intake as compared to phosphorus intake becomes still more impressive if the contrary cases are examined critically. Two of these are from the work of Hofmeister. In the same article that author gives figures for mature sheep which are plainly incorrect. The figures for the mature and growing sheep were obtained by the same methods, and it is justifiable, therefore, to doubt the adequacy of the experimental methods. A satisfactory criticism of Hofmeister's work is given by Neumann.[13]

Six of the other cases in which the calcium assimilation was not deficient occur in the work of Forbes, Beegle, Fritz, and Mensching on pigs. The pigs in question had been for 51 days on rations which contained from five to ten times as much phosphorus as calcium. Their calcium and phosphorus assimilation was followed during this period, and the calcium assimilation was always observed to be markedly deficient with respect to the phosphorus. At the end of the 51 days they were changed to a "meat meal" or skim milk ration with a comparatively high calcium content, and it is under these circumstances that the $\frac{\text{Ca}}{\text{P}}$ ratio becomes a little larger than the expected one.

Four more cases in which the calcium assimilation was not deficient with respect to the phosphorus are those of Sawyer, Baumann, and Stevens in their work on human beings. In this work, however, the phosphorus balances were negative throughout; and the results, therefore, cannot be taken as representing anything approaching average conditions in growing human beings.

O., *Landw. Ver.-Stat.*, 1904–05, lxi, 451. Köhler, A., Honcamp, F., and Eisenkolbe, P., *ibid.*, 1907, lxv, 349. Forbes, E. B., with collaborators, *Ohio Agric. Exp. Station, Techn. Bull. 6*, 1915. Forbes, E. B., Beegle, F. M., Fritz, C. M., and Mensching, J. E., *Ohio Agric. Exp. Station, Bull. 271*, 1914. Sawyer, M., Baumann, L., and Stevens, F., *J. Biol. Chem.*, 1918, xxxiii, 103.

[12] Weiske, H., *Landw. Jahrb.*, 1880, ix, 205.

[13] Neumann, J., *J. Landw.*, 1893, xli, 343.

Out of the 136 individual experiments on growing animals, therefore, the calcium intake is deficient with respect to the phosphorus in 118; and of the eighteen which do not exhibit this peculiarity, two are open to grave suspicion of experimental error, six were carried out under conditions which quite unusually favored an excessive calcium intake, and four are of such nature that they cannot be regarded as representing growth at all.

Of the remaining six, two occur in the work of Weiske. Lambs were kept on a fairly uniform ration between the ages of 4 and 14 months, and 8 day balance periods were run at intervals of about a month. If the conditions under which animals are put in balance experiments do not disturb calcium and phosphorus metabolism, this procedure ought certainly to give a representative picture of the manner in which these elements are assimilated between the ages of 4 and 14 months, in lambs.

The whole experiment falls readily into two main periods. The first of these lasted a little more than 2 months, and in it the ratio $\frac{\text{Ca assimilation}}{\text{P assimilation}}$ was a little larger than is to be expected. The second period includes the last $7\frac{1}{2}$ months of the experiment, during which the ratio $\frac{\text{Ca assimilation}}{\text{P assimilation}}$ was decidedly smaller than the expected one. The markedly low $\frac{\text{Ca}}{\text{P}}$ ratios during the last $7\frac{1}{2}$ months much more than compensate for the slightly high ones during the first $2\frac{1}{2}$ months; so that, if the figures were representative, the lambs' bodies at the end of the experiment would have contained much less calcium in proportion to phosphorus than is normal.

In view of the many chances of error to which balance experiments are subject, it seems to us remarkable that they should agree so nearly universally in any particular; and we think that the results point unmistakably to the conclusion that calcium assimilation is interfered with by the conditions to which animals have been subjected in these experiments.

We have studied with interest the experiments in which calcium and phosphorus metabolism have been followed in mature animals not subject to any special demand for these elements, and in mature milking animals. We do not think it worth while to give

any detailed discussion of the first of these classes of experiments. As was to have been expected, both calcium and phosphorus balances are sometimes positive and sometimes negative, but the figures are usually small, and there is no very marked general tendency for either element to be gained or lost faster than the other. It does not seem to us that these results have any bearing, one way or the other, on the view which we have gained from our study of the results on growing animals. In mature animals which are neither pregnant nor lactating, the assimilative requirements for calcium and phosphorus are small in comparison to those met by the young animal and by the pregnant or lactating female; and it is not to be expected that the mildly unusual conditions which accompany a well conducted balance experiment could show themselves very definitely as a reduction in calcium assimilation.[12, 14] But the results which have been obtained from milking animals seem to us to lend strong support to the view that the conditions of balance experiments interfere with the absorption[15] of calcium and to be incomprehensible on any other basis.

We are familiar with experiments by three independent sets of investigators, in which the calcium and phosphorus balances were followed in milking cows.[1, 16] In two of these investigations, namely, those of Anger and Forbes, most of the cows were fed on what might be called normal average rations, and the results can be conveniently considered together. Both calcium and phosphorus balances were generally negative, and the authors conclude that it is usual for milking cows to be taking these elements from their bodies in order to put them into the milk.

It is natural to suppose that the calcium and phosphorus lost by the cows in Anger's and in Forbes' experiments came from the bones. The calcium must have come chiefly from the bones, for

[14] Experiments of this kind have been carried out by Hofmeister, V., *Landw. Ver.-Stat.*, 1873, xvi, 343. Wellman, O., *Arch. ges. Physiol.*, 1908, cxxi, 508. Sherman, H. C., Gillett, L. H., and Pope, H. M., *J. Biol. Chem.*, 1918, xxxiv, 373. Sherman, H. C., Wheeler, L., and Yates, A. B., *ibid.*, 383.

[15] We think it better to use the word "absorption" for milking animals, and to reserve the term "assimilation" for the cases where the absorbed material is actually retained in the body.

[16] Anger, A., Inaugural dissertation, Bonn, 1898. Hart, E. B., McCollum, E. V., and Humphrey, G. C., *Am. J. Physiol.*, 1909, xxiv, 86.

the calcium content of the mammalian soft tissues is so small that the subjects of these experiments often lost in 3 or 4 days more calcium than is contained in the whole body of a cow outside of her bones and teeth.

The $\frac{Ca}{P}$ ratio in bone is about 2.15. But the average daily calcium loss divided by the average daily phosphorus loss in Anger's and in Forbes' experiments deviates largely from this ratio. In Anger's experiments it is 12.23; and in the three sets of experiments reported by Forbes[1] it is 9.07, 213.00, and 3.62 respectively. The calcium loss is largely in excess of what it should be if the process is simply a wasting of bone tissue.

The results are very difficult to reconcile with our results, which appear to show that pregnant cows readily absorb enough phosphorus from their food to supply the growing embryo, but that they are usually compelled to supply calcium to their offspring from their own bones. As many cows are always either pregnant or milking, or both, through a large proportion of their lives, these results taken together would seem to point to the impossible conclusion that the normal milch cow is constantly changing the $\frac{Ca}{P}$ ratio of her body in the direction of more phosphorus and less calcium throughout the whole period of her useful life.

The experiment of Hart, McCollum, and Humphrey has been included in this discussion because of the very surprising nature of the conclusions to be drawn from it on the supposition that the calcium and phosphorus metabolism of cows is not disturbed by the collection of their excreta. A single milking cow was kept for 110 consecutive days on known rations low in calcium, and the excreta were collected during 25 days of this period so that the calcium and phosphorus balances could be followed. If it is supposed that the results obtained in the collection periods can be applied to the whole 110 days, it would follow that during this period the cow lost about 25 per cent of all the calcium contained in her body and remained very nearly in phosphorus equilibrium. We should have to suppose, therefore, either that the composition of her bones was profoundly altered, or that 25 per cent of the phosphorus lost from the bones along with the calcium was stored in the soft tissues.

Weiske[17] has studied the bones of a milking goat which was kept for 50 days on a low calcium ration. His results show that the change in the $\frac{Ca}{P}$ ratio of the bones produced by this treatment is negligible. And Hart, McCollum, and Fuller[18] have furnished excellent evidence against the possibility of producing any considerable changes in the phosphorus content of the mammalian soft tissues. It seems to us, therefore, that the experiment of Hart, McCollum, and Humphrey[16] can best be interpreted by supposing that their figures for calcium and phosphorus metabolism during the collection periods are not representative for the whole 110 days of the experiment, and that in the intermediate periods the cow largely made up the marked calcium deficiency produced during the collection periods.

The situation may be summed up as follows. There is an apparent contradiction between the results obtained regarding the calcium and phosphorus balances of growing animals and what is known regarding the calcium and phosphorus content of the bodies of the same species of animals. There is a similar contradiction between results that have been obtained regarding the calcium and phosphorus balances of milking animals, and results obtained from studying the bones of milking animals. Finally, the results which have been obtained regarding the calcium and phosphorus balances of milking cows appear to be irreconcilable with those which have been obtained regarding those balances in cows toward the end of pregnancy. All these difficulties would be removed by making the assumption that the conditions to which animals have been subjected in balance experiments are likely to interfere with calcium absorption.

We are the more ready to make this assumption, because it is in full agreement with recent striking scientific evidence in regard to the effects of emotional disturbance on the digestive processes. It has been shown by numerous investigators that comparatively mild disagreeable stimuli are capable of producing disturbances in the nervous system which may inhibit the secretion of the

[17] Weiske, H., *Z. Biol.*, 1871, vii, 179, 333.

[18] Hart, E. B., McCollum, E. V., and Fuller, J. G., *Univ. Wisconsin Agric. Exp. Station, Research Bull. 1*, 1909.

digestive juices and the movements of the alimentary tract for considerable periods. A good review of the subject with references to the literature is given by Cannon.[19]

Our Own Evidence in Regard to Disturbance and Calcium Assimilation.

Throughout our own experiments, two 4 day collection periods followed each other without any change at all in the experimental routine; and in each case, except that of No. 66, the two collection periods followed a preliminary 10 day period without any change in routine except the introduction of the collection of urine and feces. If the collection of urine and feces produced no disturbance in metabolism, the metabolic behavior of the cows should have been the same in each case in the first of the 4 day collection periods as in the immediately following one; and the same, as far as it was followed, in the preliminary periods as in the collection periods. This was not the case.

It has been our experience that any slight change in the surroundings of a cow or disturbance of her habits is likely to cause a reduction in the amount of water which she will drink. The water taken by Nos. 51 and 63 in the collection periods, March 3 to 10, in the intermediate period, March 11 to 20, and in the collection periods, March 21 to 28, was followed (Table II). Both cows took decidedly more water in the intermediate period than in any of the collection periods.

Much more evidence pointing in the same direction can be obtained by comparing the metabolic processes of the cows in the first collection periods and in the immediately succeeding ones. But, before discussing this evidence, it must be pointed out that certain accidents occurred in the course of the experiments which must be taken into account in interpreting the results.

No. 63 developed abrasions during the preliminary period, February 17 to March 2, and refused to lie down during the first 60 hours of the collection period, March 3 to 6. On the evening of March 5, she was provided with a softer bed, and there was no trouble after that. Further, No. 51 could not be used in

[19] Cannon, W. B., Bodily changes in pain, hunger, fear and rage, New York and London, 1915, Chapter I, 1–20.

the experiment after March 21; No. 66 was unexpectedly substituted for her, and went through the two collection periods, March 21 to 24 and 25 to 28, without any preliminary period in the experimental surroundings. In her case, therefore, the additional factor of new surroundings was added to that of the mere collection of urine and feces.

Table II shows that No. 51 drank more water from March 3 to 6 than from March 7 to 10; and No. 63, more from March 21 to 24 than from March 25 to 28. These results suggest that the collection of urine and feces produced more disturbance during the second of the two consecutive 4 day collection periods than during the first one. It is true that No. 63 drank more water from March 7 to 10 than from March 3 to 6; and No. 66, more from March 25 to 28 than from March 21 to 24. But neither of these results is really out of harmony with the suggestion indicated above. In the case of No. 63 an additional disturbing factor was introduced during the period March 3 to 6 by the fact that the abrasions which had developed on her hips and shoulders prevented her from lying down. The case of No. 66, on the other hand, may well be discarded from the discussion, for, in addition to the fact that she had had no preliminary period in the experimental stall, the water which she drank on March 21 could not be determined.

The manner in which our cows took their water, therefore, suggests that a disturbance was produced by the collection of their urine and feces, which was cumulative for a considerable period and produced more marked effects in the second 4 days of collection than in the first 4. This suggestion is strongly confirmed by a study of the nitrogen, calcium, and phosphorus balances given in Table X. In every case, except that of No. 63, March 3 to 10, where the exceptional disturbance was accidentally introduced in the first 4 days, the assimilation of all three elements occurred more rapidly in the first of the 4 day collection periods than in the immediately succeeding one. In the case of No. 63, March 3 to 10, the relations are reversed for all three elements.

It seems surprising perhaps at first sight that assimilation was generally better in the first than in the second 4 day collection period. It might have been expected that the cows would be more accustomed to the collection of their excreta in the second period, and that their assimilative processes would therefore

have been more nearly normal. But we can see no escape from the facts, and the circumstance that the relations are reversed in the case of No. 63, March 3 to 10, seems to us decidedly to strengthen the case. Steenbock and Hart[20] have reported results which show how persistent may be the tendency toward a negative calcium balance under the conditions of a balance experiment, and how this tendency may be overcome by a short period of relief from these conditions.

Our results are in agreement with those of past experiments in showing that the assimilation of calcium is more quickly and more profoundly affected by the experimental procedure than that of either phosphorus or nitrogen. It has already been pointed out that the phosphorus assimilation in Nos. 51 and 63 was about enough on the average to supply the phosphorus needed by their growing unborn calves, but that the calcium assimilation was never sufficient for this in any single period. It must be pointed out in addition that the fluctuations in calcium assimilation as between the first and second 4 day periods were greater both relatively and absolutely in every case than the similar fluctuations for either phosphorus or nitrogen.

The Effects on Calcium, Phosphorus, and Nitrogen Assimilation of Feeding Sodium Phosphate and Alternating the Grain and Hay Rations.

For convenience the rations fed to No. 63, March 3 to 10, and to No. 66, March 21 to 28, will be called the "experimental rations," while the rations fed to No. 51, March 3 to 10, and to No. 63, March 21 to 28, will be called the "control rations."

The results indicate that phosphorus assimilation is favored by feeding the experimental rations. Although it is clear from certain aspects of the results that phosphorus assimilation is interfered with by disturbance, and that the cows on the experimental rations were decidedly more disturbed than those on the control rations, nevertheless, the cows fed the experimental rations assimilated phosphorus uniformly more rapidly than the controls.

In regard to calcium assimilation the results are not so consistent but on the whole they favor the view that this process also occurs

[20] Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, 1913, xiv, 59.

more rapidly in the cows fed the experimental rations. It is true that the most rapid calcium assimilation occurs in the case of No. 51, March 3 to 10, on the control ration. But this cow was decidedly less disturbed by the experimental conditions than any of the others, and she was, in addition, the largest, the most robust, and the best milker of the three. All the other results are consistent in indicating that calcium assimilation occurs more rapidly during the experimental feeding. No. 63, for instance, was assimilating calcium more rapidly during the experimental period, March 3 to 10, than during the control period, March 21 to 28, although she was much more disturbed by the experimental procedure during the first than during the second of these periods; and even No. 66, though she was not carrying a calf, and was evidently much upset by the double disturbance of new surroundings and collection of her urine and feces during the period March 21 to 28, nevertheless, assimilated calcium more rapidly on the experimental rations during this period than did No. 63 during the same period on the control rations.

The results appear to indicate that the experimental feeding is unfavorable to nitrogen assimilation. But we have kept cows for long periods on the experimental rations, and have found that they gained weight at least as well and remained in at least as good general condition as others on the control rations (page 471). We do not think, therefore, that the experimental rations are unfavorable to nitrogen assimilation, and we are inclined to attribute the reduced nitrogen assimilation of No. 63 on these rations to the facts that, when on these rations, she was at an earlier stage of pregnancy and more disturbed by the experimental procedure. That No. 66 should have assimilated nitrogen as fast as the other two cows is not to be expected, as she was not carrying a calf; and the fact that her nitrogen balance was positive at all indicates that the experimental feeding is not unfavorable to nitrogen assimilation.

Calcium and Phosphorus in Food, Blood, and Urine.

In Table XII we have brought together our results on urine and on blood plasma collected approximately simultaneously. The results there given indicate that there is a connection between

the phosphate feeding, the concentration of inorganic phosphorus in the blood plasma, and the amount of phosphorus excreted in the urine. No. 63, for instance, during the period March 3 to 10, when she was fed phosphate, had an average concentration of about 0.0063 per cent phosphorus in her plasma and was excreting about 0.88 gm. daily in her urine. During the period March 21 to 28, when she received no phosphate, her plasma phosphorus dropped to 0.0052 per cent and her daily urinary phosphorus excretion to about one-ninth of what it was previously. No. 51, March 3 to 10, received no phosphate, had a plasma phosphorus of about 0.0056 per cent, and was excreting less than 0.1 gm. of phosphorus in her daily urine.

Closer examination of the figures, however, shows that the urinary excretion of phosphorus does not depend entirely on the concentration of inorganic phosphorus in the plasma. No. 66, March 21 to 28, for instance, had a decidedly higher plasma phosphorus than either No. 51, March 3 to 10, or No. 63, March 21 to 28, and was, nevertheless, excreting less phosphorus in her daily urine. And if the figures given for No. 63 for February 17, March 3, and March 11 are examined, it will be found that there is an inverse relation between the plasma phosphorus and the daily urinary phosphorus excretion.

We are inclined to suspect that these disturbances of the relation between the concentrations of plasma phosphorus and urinary phosphorus were connected with differences in the acid-base equilibrium of our animals; but, in the present inadequate state of our knowledge, we do not think it worth while to discuss this matter further.

We obtained wide variations in the amount of calcium excreted in the urine, in spite of the fact that all our cows at all times received about the same amount of calcium in their rations, and that there was little variation in the concentration of calcium in their blood plasma. In looking for an explanation for these variations, we have been struck by the fact that there is an inverse relation between the concentrations of urinary calcium and phosphorus in our experiments, and we think that this circumstance has some significance. Bertram[4] and Rüdel[21] have reported

[21] Rüdel, G., *Arch. Exp. Path. u. Pharmacol.*, 1894, xxxiii, 79.

results which indicate a tendency toward inverse relationship between the calcium and phosphorus of mammalian urine, and it is of course impossible that an alkaline fluid such as average cow's urine could hold much calcium and phosphate in solution at the same time.

But we do not think it worth while to discuss the mechanism of these relationships at the present time. The chief value of the results which we are considering lies in the demonstration that the amount of calcium excreted in the urine is largely independent of the concentration of calcium in the blood plasma.

SUMMARY.

1. The separate collection of urine and feces by attendants, as practiced in balance experiments on cows, produces a nervous disturbance in the animals which interferes markedly with the assimilation of calcium, and, to a less degree, with that of nitrogen and phosphorus. A critical examination of the results of balance experiments carried out in the past indicates that, in the great majority of instances, the experimental procedure has interfered with calcium assimilation.

2. The assimilation of phosphorus by pregnant cows, and probably that of calcium also, is favored by adding disodium phosphate to the grain and feeding the grain and hay of the ration on alternate days.

3. The urinary excretion of phosphorus is markedly influenced by the concentration of inorganic phosphorus in the blood plasma. But our results show that it is influenced also by another factor, which may be connected with the acid-base equilibrium of the body.

4. In our experiments there has been an inverse relation between the amounts of calcium and phosphorus excreted in the urine. But we have no doubt that this relation is easily disturbed by other influences, particularly the relation between the acids and bases of the ration.

TABLE I.

Weights of Cows.

Date.	No. 51.	No. 63.	Date.	No. 51.	No. 63.
1919	*kg.*	*kg.*	*1919*	*kg.*	*kg.*
Feb. 14	529.5	478.6	Mar. 18	540.9	462.3
" 15	515.5	481.4	" 19	542.6	461.4
" 16	514.1	479.1	" 20	550.0	461.4
Average.....	519.7	479.7	.....	544.5	461.7
Feb. 26	524.1	465.5			
" 27	529.1	465.9			
" 28	525.9	467.7			
Average.....	526.4	466.4			
				No. 66.	
Mar. 11	540.5	461.4	Mar. 29	397.7	481.8
" 12	542.7	460.0	" 30	408.6	476.8
" 13	538.6	461.4	" 31	401.8	473.6
Average.....	540.6	469.9	.....	402.7	477.4

TABLE II.

*Water Drunk by Cows.**

Date.	No. 51.	No. 63.	Date.	No. 51.	No. 63.
1919	*kg.*	*kg.*	*1919*	*kg.*	*kg.*
Mar. 3	20.41	19.50	Mar. 19	26.62	20.41
" 4	16.19	5.10	" 20	21.63	20.01
" 5	20.95	12.22			
" 6	21.60	17.55			
Average	19.79	13.59		23.88	21.07
				No. 66.	
Mar. 7	16.92	20.13	Mar. 21	Undetermined.†	16.87
" 8	0.00	20.07	" 22	0.00	16.36
" 9	18.65	6.38	" 23	13.89	24.58
" 10	13.55	23.95	" 24	7.85	16.84
Average	12.28	17.63		7.25‡	18.66
Mar. 11	13.58	17.86	Mar. 25	18.82	9.07
" 12	31.35	18.09	" 26	16.78	27.47
" 13	27.07	23.22	" 27	13.15	15.88
" 14	21.94	20.04	" 28	12.02	17.97
" 15	26.11	24.55			
" 16	22.96	21.43			
" 17	26.42	25.46			
" 18	21.12	19.67			
Average				15.19	17.60

* The water supplied to the cows was tap water. Samples of it were analyzed for calcium and phosphorus, and it was found to contain 0.9 mg. of calcium per liter, and only a trace of phosphorus. The phosphorus content was certainly less than 0.005 mg. per liter. As none of the cows ever took as much as 30 liters of water in a day, the calcium and phosphorus which they got from the water may be disregarded in calculating the balances.

† No. 66 had free access to a water trough during the early morning of March 21, and in the afternoon she drank 10.55 kg. of water.

‡ Average calculated from water drunk during last 3 days of test.

TABLE III.

*Feed Offered to Cows.**

Date.	No. 51.			No. 63.		
	Grain without phosphate.†	Alfalfa hay.	Corn silage.	Grain with phosphate.‡	Alfalfa hay.	Corn silage.
1919	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Feb. 17	1,361	1,814	13,608	0	3,628	13,608
" 18	1,361	1,814	13,608	2,722	0	13,608
" 19	1,361	1,814	13,608	0	3,628	13,608
" 20	1,361	1,814	13,608	2,722	0	13,608
	Same till Mar. 1.			Same till Mar. 1.		
Mar. 1	1,361	1,814	13,608	0	3,628	5,443
" 2	1,361	1,814	13,608	2,722	0	10,886
" 3	1,361	1,814	13,608	0	3,628	10,886
	Same till Mar. 11.			Same till Mar. 11.		
Date.	Grain with phosphate.‡	Alfalfa hay.	Corn silage.	Grain without phosphate.†	Alfalfa hay.	Corn silage.
1919	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Mar. 11	0	3,628	13,608	1,361	1,814	10,886
" 12	2,722	0	13,608	1,361	1,814	10,886
	Same till Mar. 21.			Same till Mar. 21.		
Date.	No. 66.			No. 63.		
	Grain with phosphate.‡	Alfalfa hay.	Corn silage.	Grain without phosphate.†	Alfalfa hay.	Corn silage.
1919	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Mar. 21	0	3,628	13,608	1,361	1,814	10,886
" 22	2,722	0	10,886	1,361	1,814	10,886
" 23	0	3,628	10,886	1,361	1,814	10,886
	Same till Mar. 29.			Same till Mar. 29.		

* From Jan. 31 to Feb. 16 inclusive No. 51 had received the same ration as that given Feb. 17 to Mar. 11; No. 63, from Feb. 3 to 16, the same as from Feb. 17 to 28; No. 66, from Dec. 18, 1917, to Mar. 20, 1919, inclusive, the same as from Mar. 21 to 28, except that throughout the earlier period her daily silage was 13,608. All three cows had eaten satisfactorily the rations they received before being put on the experiment.

† Grain without phosphate composed of 50 parts of corn and cob meal, 40 parts of wheat bran, 20 parts of cottonseed meal, and 1 part of NaCl.

‡ Grain with phosphate composed of 50 parts of corn and cob meal, 40 parts of wheat bran, 20 parts of cottonseed meal, 1 part of NaCl, and 10 parts of Na_2HPO_4 with about 9 molecules of water of crystallization.

TABLE IV.

*Feed Refused by Cows; Totals for Periods.**

Period.	No. 51.	No. 63.
1919		
Feb. 17–Mar. 2	0	Approximately 900 gm. of grain, 100 gm. of hay, 4,000 gm. of silage.
Mar. 3–6	0	6,237 gm. of a mixture of hay and silage.
" 7–10	0	Approximately1 ,300 gm. of a mixture of hay and silage.
" 11–20	0	Negligible quantities; not weighed.
	No. 66.	No. 63.
Mar. 21–24	Approximately 3,000 gm. of pieces of cob and stem.	Approximately 300 gm. of a mixture of hay and silage.
" 25–28	Approximately 2,500 gm. of pieces of cob and stem.	Approximately 1,200 gm. of a mixture of hay and silage.

*The feed refused was, in most cases, dried without being weighed. There was no object in obtaining its moisture content as it had always lain for some time in the manger, and contained, therefore, a different amount of moisture from the feed originally offered. The dried refused feed was weighed and analyzed for nitrogen, calcium, and phosphorus (Table VI). The figures give a fairly accurate idea of the proportions of grain, hay, and silage contained in the various samples of feed refused.

TABLE V.

Composition of Materials Fed.

Feed.		Water.	Nitrogen.	Calcium.	Phosphorus.
		per cent	*per cent*	*per cent*	*per cent*
Grain with phosphate		17.59	2.1036	0.0659	1.4727
" without "		13.84	2.1837	0.0707	0.7007
Hay		16.73	1.8754	1.3103	0.2138
Silage	Mar. 3–6	66.91	0.3951	0.1035	0.0733
	" 7–10	69.21	0.3400	0.0926	0.0650
	" 21–24	69.98	0.3321	0.1001	0.0671
	" 25–28	67.45	0.3102	0.1037	0.0686

TABLE VI.

Nitrogen, Calcium, and Phosphorus in Feed Refused. Absolute Quantities of Elements in Daily Average Quantities of Feed Refused.

Cow No.	Period.	Nitrogen.	Calcium.	Phosphorus.
	1919	*gm.*	*gm.*	*gm.*
63	Mar. 3–6	8.99	3.4975	1.5328
	" 7–10	2.38	1.0963	0.4866
	" 21–24	0.69	0.3566	0.0991
	" 25–28	2.75	1.4264	0.3962
66	Mar. 21–24	2.71	0.7559	0.6819
	" 25–28	1.81	0.6218	0.5770

TABLE VII.

Absolute Quantities of Feces and Urine Voided during Collection Periods.

Date.	No. 51.		No. 63.		Date.	No. 66.		No. 63.	
	Feces.	Urine.	Feces.	Urine.		Feces.	Urine.	Feces.	Urine.
1919	*gm.*	*gm.*	*gm.*	*gm.*	*1919*	*gm.*	*gm.*	*gm.*	*gm.*
Mar. 3	19,674	4,593	15,422	4,352	Mar. 21	13,749	6,124	13,919	3,515
" 4	18,031	4,905	17,151	4,678	" 22	10,801	3,147	15,138	3,487
" 5	16,188	5,103	11,879	4,649	" 23	11,992	4,253	17,549	3,260
" 6	13,721	4,536	12,786	3,572	" 24	13,580	3,062	17,436	3,175
Total.....	67,614	19,137	57,238	17,251	.....	50,122	16,586	64,042	13,437
Average.....	16,903	4,784	14,309	4,313	.....	12,530	4,146	16,010	3,359
Mar. 7	15,082	4,196	17,436	0	Mar. 25	13,636	3,742	20,355	3,600
" 8	14,260	4,990	15,196	6,861	" 26	11,850	3,544	18,257	3,969
" 9	14,571	4,593	17,123	3,062	" 27	11,312	4,026	15,649	3,856
" 10	14,006	6,832	16,642	3,317	" 28	12,219	3,544	18,285	1,673
Total.....	57,919	20,611	66,397	13,240	.....	49,017	14,856	72,546	13,098
Average.....	14,480	5,153	16,599	3,310	.....	12,254	3,714	18,136	3,274

TABLE VIII.

Composition of Moist Feces.

Period.	No. 51.				No. 63.			
	Water.	Nitrogen.	Calcium.	Phosphorus.	Water.	Nitrogen.	Calcium.	Phosphorus.
1919	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Mar. 3–6	85.86	0.2950	0.2037	0.1178	85.45	0.3212	0.2200	0.1820
" 7–10	85.09	0.3095	0.2438	0.1390	86.63	0.2912	0.1925	0.1560
	No. 66.				No. 63.			
Mar. 21–24	83.84	0.3441	0.2393	0.1881	86.19	0.2921	0.2017	0.1004
" 25–28	82.66	0.3608	0.3043	0.2436	85.91	0.2839	0.1984	0.1017

TABLE IX.

Composition of Urine.

Period.	No. 51.				No. 63.			
	Specific gravity.	Nitrogen.	Calcium.	Phosphorus.	Specific gravity.	Nitrogen.	Calcium.	Phosphorus.
1919		per cent	per cent	per cent		per cent	per cent	per cent
Mar. 3–6	1.037	1.0824	0.0183	0.0019	1.038	1.0832	0.0007	0.0208
" 7–10	1.035	0.9928	0.0183	0.0016	1.038	1.3196	0.0009	0.0263
	No. 66.				No. 63.			
Mar. 21–24	1.042	1.2311	0.0121	0.0020	1.037	1.0789	0.0016	0.0032
" 25–28	1.045	1.4203	0.0090	0.0021	1.036	0.9352	0.0013	0.0031

TABLE X.

Average Daily Intake, Output, and Balance of Nitrogen, Calcium, and Phosphorus.

Cow No.	Period.	Nitrogen.	Calcium.	Phosphorus.
		Food. Feces. Urine. Balance.	Food. Feces. Urine. Balance.	Food. Feces. Urine. Balance.
	1919	*gm.*	*gm.*	*gm.*
		117.50	38.81	23.39
51	Mar. 3–6	49.87	34.43	19.91
		51.78	0.88	0.09
		+15.85	+3.50	+3.39
		110.01	37.33	22.27
51	Mar. 7–10	44.81	35.30	20.13
		51.16	0.94	0.08
		+14.04	+1.09	+2.06
		96.66	32.42	30.37
63	Mar. 3–6	45.96	31.48	26.04
		46.72	0.03	0.90
		+3.98	+0.91	+3.43
		97.29	33.63	30.51
63	Mar. 7–10	48.34	31.95	25.89
		43.68	0.03	0.87
		+5.27	+1.65	+3.75
		98.36	35.47	30.99
66	Mar. 21–24	43.12	29.99	23.57
		51.04	0.50	0.08
		+4.20	+4.98	+7.34
		94.61	35.32	30.85
66	Mar. 25–28	44.21	37.29	29.85
		52.75	0.33	0.08
		−2.35	−2.30	+0.92
		99.20	35.27	20.61
63	Mar. 21–24	46.77	32.29	16.07
		36.24	0.05	0.11
		+16.19	+2.93	+4.43
		94.76	34.59	20.49
63	Mar. 25–28	51.49	35.98	18.45
		30.62	0.04	0.10
		+12.65	−1.43	+1.94

TABLE XI.

Composition of Blood Samples.

Cow No.	Date.	Total blood P per 100 gm.	Blood corpuscles.	Plasma.			
				Phosphorus.			Calcium per 100 gm.
				Total per 100 gm.	Lipoid* per 100 gm. calculated.	Inorganic per 100 gm.	
	1919	*mg.*	*vol. per cent*	*mg.*	*mg.*	*mg.*	*mg.*
	Feb. 17	15.7	34.6	9.9	4.9	5.0	Not determined.
51	Mar. 3	15.9	34.1	10.1	4.3	5.8	" "
	" 11	15.3	32.5	9.6	4.2	5.4	9.5
	Feb. 17	18.3	38.9	12.3	5.2	7.1	Not determined.
	Mar. 3	18.2	41.0	11.5	5.3	6.2	" "
63	" 11	17.4	40.4	11.9	5.4	6.5	9.7
	" 21	17.2	39.4	9.6	4.4	5.2	9.6
	" 29	17.5	42.6	10.3	5.0	5.3	9.5
	Mar. 21	18.1	40.4	11.9	6.0	5.9	10.1
66	" 29	17.9	45.8	12.1	6.1	6.0	9.7

*Lipoid phosphorus calculated by subtracting inorganic from total (Meigs, E. B., Blatherwick, N. R., and Cary, C. A., *J. Biol. Chem.*, 1919, xxxvii, 10–17).

TABLE XII.

*Calcium and Phosphorus in Urine and Blood Plasma.**

Cow No.	Date.	Plasma.		Urine.			
		Calcium.	Inorganic phosphorus.	Calcium.	Phosphorus.	Daily calcium excretion.	Daily phosphorus excretion.
	1919	*per cent*	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*
	Feb. 17	Not determined.	0.0050	Not determined.	0.0017	Not determined.	0.0689
51	Mar. 3	" "	0.0058	0.0183	0.0019	0.8755	0.0907
	" 11	0.0095	0.0054	0.0183	0.0016	0.9425	0.0818
	Feb. 17	Not determined.	0.0071	Not determined.	0.0099	Not determined.	0.3817
	Mar. 3	" "	0.0062	0.0007	0.0208	0.0303	0.8979
63	" 11	0.0097	0.0065	0.0009	0.0263	0.0293	0.8706
	" 21	0.0096	0.0052	0.0016	0.0032	0.0543	0.1093
	" 29	0.0095	0.0053	0.0013	0.0031	0.0423	0.1006
66	Mar. 21	0.0101	0.0059	0.0121	0.0020	0.5000	0.0827
	" 29	0.0097	0.0060	0.0090	0.0021	0.3340	0.0774

*In this table the concentrations of calcium and phosphorus in the blood plasma are taken as corresponding to the concentrations in the urine of the nearest collection period. For instance, the plasma obtained Mar. 3 is taken as corresponding to the urine collected in the period Mar. 3–6; the plasma obtained Mar. 11, to the urine collected Mar. 7–10; and so on. On Feb. 17, 18 hour samples of urine were specially collected to compare with the blood samples obtained on that date.

FAT-SOLUBLE VITAMINE.*

II. THE FAT-SOLUBLE VITAMINE CONTENT OF ROOTS, TOGETHER WITH SOME OBSERVATIONS ON THEIR WATER-SOLUBLE VITAMINE CONTENT.

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Plate 1.

(Received for publication, October 24, 1919.)

In the evolution of the modern conception of the dietary needs of the body there has come an appreciation of the significance of small amounts of those indispensable nutritive factors known as vitamines; but unfortunately with this appreciation there has been aroused much apprehension in the minds of many in regard to the wisdom of omitting certain foods from the human diet for fear of unknowingly reducing the vitamine content of the diet to dangerous limits. While it is not intended to deprecate the value of discretion in the selection of the ingredients of the diet for reasons other than satisfying the requirements of palatability, good proteins, and mineral elements, it is deemed rather unfortunate that conclusions should have been hastily drawn and promiscuously applied after only a limited survey of the dietary properties of our naturally occurring foods had been made, and especially when such were made with animals about which we have no information as to their requirements when compared with those of man. Taking into consideration the apparent limitations imposed on methods of experimental inquiry now in use, what seems most desirable above all else—after the development of sufficient theory to serve as a suitable working basis—is the accumulation of sufficient data which may give an insight into the specific comparative nutritive properties of dif-

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ferent foods. Such information seems especially called for by the great interest shown by dietitians in the occurrence and stability of the vitamines.

It is now generally recognized that of the substances which are collectively known as vitamines there are three distinct kinds; namely, the water-soluble or antineuritic vitamine, the fat-soluble or antixerophthalmic vitamine, and the antiscorbutic vitamine. In the present series of papers it is planned to confine the discussion to the fat-soluble vitamine, but incidentally certain points of interest in connection with the water-soluble vitamine will be mentioned, especially with reference to the determination of the amount present so that it could not possibly be a limiting factor in the performance of the animals on any of the rations which it was desired to test for the relative fat-soluble vitamine content. Various phases of the water-soluble vitamine question will be published in another connection.

The specific growth-promoting property which is now associated with the occurrence of the fat-soluble vitamine was first dissected from different factors, though functioning in a similar capacity, when McCollum and Davis[1] in 1913, in a study of the value of different salt mixtures, observed that in a synthetic ration an ether extract of butter or egg yolk had a stimulating action on growth which was not possessed by other fats such as lard or olive oil. Very shortly thereafter Osborne and Mendel[2] published experiments, some of them of 180 days duration, where it was pointed out that their milk rations had special dietary properties not found in their milk-free rations and that this characteristic seemed to be true of rations carrying an equivalent amount of butter as well. Later[3] they demonstrated in very conclusive experiments, more so than any published up to that time. that this property resided in the butter fat. Furthermore, they substantiated McCollum and Davis' observation that egg yolk fat was efficient in this respect and added that "some other oils" were no more efficient than lard. In 1914 Osborne and Mendel[4] reported

[1] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167.
[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xv, 311.
[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 423.
[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 402.

"We have likewise obtained uniform success by substituting cod liver oil for a portion of the lard in our standard diets. . . . . In contrast with this are the uniformly observed failures of almond oil to restore growth when it had ceased on the usual lard food mixtures."

It is not intended to give here a résumé of the findings of others with respect to the occurrence of the fat-soluble vitamine in fats, grains, leaves, and other food materials, as that has already been done in a most excellent manner by Osborne and Mendel[5] and by Emmett and Luros,[6] nor do we intend to comment extensively as to the merits of the recorded observations. It is, however, safe to venture that the time is not yet ripe to warrant general classification of foods into groups rich, poor, and free from fat-soluble vitamine as general methods of experimentation, especially in reference to vitamine content of the basal food ingredients, period of observation, and control of experimental animals, differ so greatly in different laboratories that the conclusions of the different observers are hardly comparable. Furthermore, there is much reason to believe, as will be brought out in later papers, that the variation in the fat-soluble vitamine content of naturally occurring food materials, even when harvested at the same stage of development, is tremendous. No doubt there lies here the foundation for many interesting correlations in the functional rôle of specific substances in both plant and animal kingdoms, the significance of which can scarcely be predicted.

One correlation that has been advanced is an apparent attempt to associate a richness in vitamine content with the occurrence of a preponderance of actively functioning cells.[7] This deduction would seem to be based on a recognition of the great indispensibility of the vitamines for growth and an assumption that the vitamines are equally indispensable in individual physiological processes, none the less active, but of a highly specialized character. Suffice it to say that as nothing of the specific internal rôle of vitamines is known outside of the pharmacological findings of Uhlmann,[8] who, however, worked with extracts

[5] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 309.
[6] Emmet, A. D., and Luros, G. O., *J. Biol. Chem.*, 1919, xxxviii, 441.
[7] McCollum, E. V., *J. Home Econ.*, 1918, x, 195.
[8] Uhlmann, F., *Z. Biol.*, 1917–18, lxviii, 419, 457.

of very complex character, such generalizations appear gratuitous. In this connection a high vitamine content of liver tissue, instead of being due to or associated with its activity as a glandular organ, might with equal propriety be assumed on the basis of its well known functions as a storage organ, or might possibly be attributed to the absorption of substances not indispensable to the organ but absorbed due to an inefficiency of the mechanism which excludes the entrance of substances in amounts greater than the needs as these substances are brought to it in the portal circulation. Similarly, the high vitamine content of the kidney may be said to be due to the temporary retention of the vitamines as unutilizable amounts of them found in the ration are eliminated.

One thing appears reasonably certain: In the plant kingdom the occurrence of the vitamine cannot always be associated with a preponderance of actively functioning cells as distinguished from those that serve primarily as storage organs. This is brought out in an analysis of our data on the occurrence of the fat-soluble vitamine in tubers and roots even though all must be considered storage organs for those constituents that are needed by the growing plant in the following season.[9] The sweet potato, as an example of the tuber, is rich in this vitamine, while the rutabaga and the potato contain relatively little of this constituent. Similarly, among the roots carrots contain much of it as has been suggested by the work of Denton and Kohman,[10] while mangels and sugar beets are poor in it. Results will be discussed in further detail under the various following experimental headings.

EXPERIMENTAL.

The relative amounts of vitamine present in the tubers and roots were determined by incorporating various percentages of them in a basal rat ration which already satisfied all other nutritive requirements, and which was as free from the vitamines as ordinary methods of laboratory manipulation could produce them. We have repeatedly demonstrated that the basal constituents of our rations were too poor in vitamines to influence in any way

[9] Steenbock, H., *Science*, 1918, xlvii, 119.

[10] Denton, M. C., and Kohman, E., *J. Biol. Chem.*, 1918, xxxvi, 249.

the conclusions at which we have arrived, but on the other hand we make no claim that very small amounts of some vitamines may still not have been present and thus may have influenced in degree—though not in character—failure in maintenance, growth, reproduction, and rearing of the young as indicated by the experimental rats. This will readily be appreciated on inspection of the charts in the various groups. When failure in growth or maintenance resulted and this was suspected as being due to a vitamine deficiency this was often incontrovertibly established by adding additional water or fat-soluble vitamine and noting the result. The water-soluble vitamine was added in the form of an alcoholic extract of ether-extracted wheat embryo evaporated on dextrin, while the fat-soluble vitamine was added as found in butter fat obtained by melting and filtering butter fat at a low temperature. The dextrin used was partially dextrinized corn-starch prepared by heating the starch with 0.1 per cent citric acid solution in an autoclave for a number of hours and then drying and grinding it. The casein was prepared from commercial casein by washing it repeatedly with distilled water acidified with acetic acid for an entire week and then drying and pulverizing it. Salts were incorporated in the rations in the form of artificial salt mixtures prepared from purified reagents.[11]

[11] The salt mixtures used had the following composition,—

Salt 35:	
$NaCl$	1.0
$CaCO_3$	1.5
Salt 1:	
$NaCl$	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2H_2O$	0.540
Fe citrate	0.118
Ca lactate	1.300
Salt 32:	
$NaCl$	0.202
$MgSO_4$(anhydrous)	0.311
$Na_2HPO_412H_2O$	0.526
K_2HPO_4	1.115
$Ca_2H_2(PO_4)_2H_2O$	1.116
Ca lactate	0.289
Fe citrate	0.138

When these experiments were initiated no data on the heat stability of the fat-soluble vitamine as found in plant materials were available. From the work of McCollum and Davis who showed that this vitamine was still contained in the yolk of hard boiled eggs[12] and from the work of Osborne and Mendel[13] on the stability of the vitamine in butter fat the idea was generally prevalent that this vitamine was thermostable. We[14] did not find this conclusion generally acceptable after we had demonstrated to our satisfaction that though the reaction is one of slow velocity the fat-soluble vitamine in butter fat is destroyed by heat. It might be mentioned that this observation has since been substantiated by Drummond.[15] As in the materials that we used, the vitamines are in contact with many different compounds and as no data on the stability of the vitamine under these conditions were available we were forced to adopt the procedure of carrying out all our drying operations at a low temperature wherever possible as the roots had to be dried for comminution and introduction into the ration. Usually the roots were dried at room temperature in an air current and later over anhydrous calcium chloride. In isolated instances, as later noted, it was necessary to use higher temperatures and in some instances it was even found necessary to cook the roots to make them digestible. Later on as our experiments progressed we found that the fat-soluble vitamine as found in plant materials was very stable to heat[16] so that we were able to allow ourselves more leeway in the variety of treatments to which the materials were subjected. Variations from the regular procedure are noted and discussed under the various experimental groups.

Fat-Soluble Vitamine in Carrots.

The roots were washed free from all extraneous material and then pulped on a power beet rasp. In this condition they dried readily in an air current at room temperature. To secure fine

[12] McCollum, E. V., and Davis, M., *Proc. Soc. Exp. Biol. and Med.*, 1913–14, xi, 101.

[13] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 381.

[14] Steenbock, H., Boutwell, P. W., and Kent, H. E., *J. Biol. Chem.*, 1918, xxxv, 517.

[15] Drummond, J. C., *Biochem. J.*, 1919, xiii, 81.

[16] Unpublished data.

comminution they were often dried over calcium chloride for a few days after which they could readily be ground to an impalpable powder in a ball mill. Usually, however, grinding in an Excelsior mill was sufficient to pulverize the pulp to a degree which prevented any picking out of the ration by the experimental animals. With the high sugar content of carrots good consumption of a high percentage carrot ration was generally secured. They were fed at levels of 5, 10, 15, 25, and 60 per cent with and without water-soluble and fat-soluble vitamine additions.

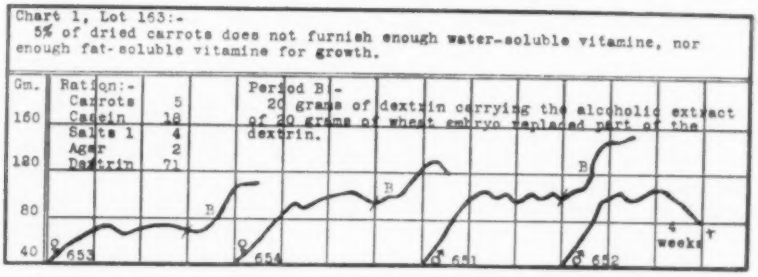


CHART 1. As seen in the chart when a ration satisfactory in all other respects is dependent for its vitamine content on 5 per cent of dried carrots there results partial failure in the growth performance which is not entirely corrected by an addition of water-soluble vitamine in the form of an alcoholic extract of ether-extracted wheat embryo. This indicates that this amount of dried carrots does not furnish a sufficiency of either the water or the fat-soluble vitamine. Rat 652 had an attack of xerophthalmia after having been on the ration 6 weeks. It was, however, but a mild attack as later during its 12th week its eyes were in good condition. At this time and for 3 weeks previously it was losing weight rapidly which appeared to be caused by the insufficiency of water-soluble vitamine as it succumbed to an acute attack of polyneuritis. Rat 653 was blinded by xerophthalmia at the end of the 10th week, but no irreparable damage was done by the acute inflammation and 2 weeks later its eyes were entirely normal. We have in this lot an instance where presence of a subnormal amount of one vitamine was apparently brought to light by a similar situation with respect to another vitamine. When the deficiency of the water-soluble vitamine was corrected the amount of fat-soluble vitamine originally present was able to allow some further growth to result. This statement can be made on the basis of results brought out later (Charts 8 to 34) which indicate that the amount of fat-soluble vitamine introduced with the water-soluble vitamine could not have been responsible for the temporary response when this was added. It brings out one of the innumerable instances where a tendency to one nutritive deficiency heightens the susceptibility and results in the onset of symptoms resulting from similar or other unfavorable environmental conditions.

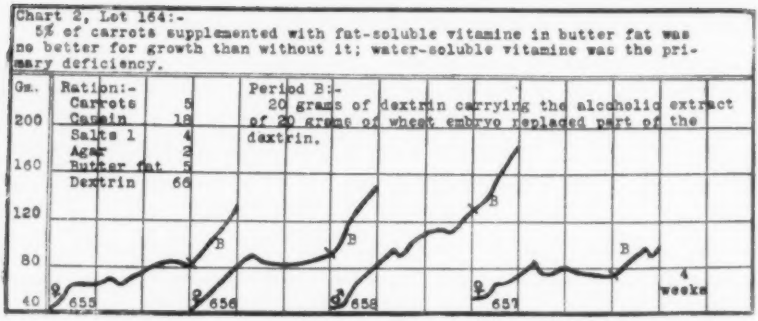


Chart 2. In this lot when the ration was already fortified with additional fat-soluble vitamine in the form of butter fat, while the growth performance was not any better than without this addition, the augmented rapidity of growth was remarkable when additional water-soluble vitamine was added as indicated in Chart 1. This shows—what had been surmised—that the primary vitamine deficiency in a ration carrying only 5 per cent of dried carrots is due to an insufficiency of the water-soluble vitamine.

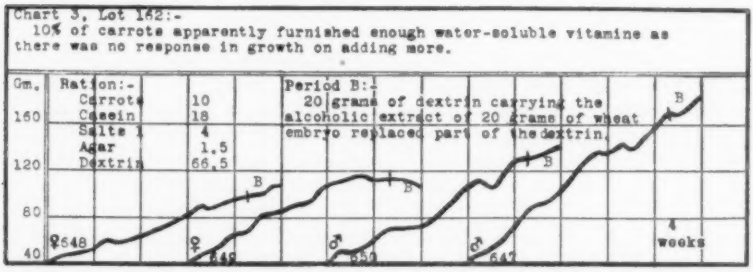


Chart 3. It appears that a ration carrying 10 per cent of dried carrots contains enough water-soluble vitamine to allow considerable growth. This is inferred from the fact that in this lot no response in augmented rapidity of growth resulted when more water-soluble vitamine was added when it was established that even in a ration carrying 5 per cent of carrots the primary deficiency was one of this vitamine. Growth here too was far from normal, but as the animals remained in fine condition during the 20 weeks of experimentation it may be taken as another instance where a ration may not be glaringly deficient in any one dietary factor, but still a suboptimal content of a number of dietetically indispensable constituents may still prevent growth from proceeding at the normal rate.

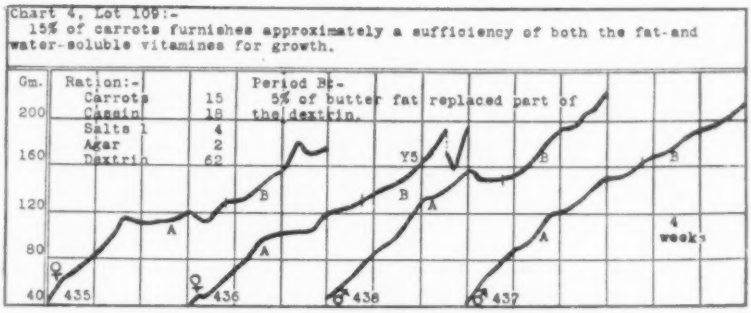


Chart 4. On a ration containing 15 per cent of carrots as the sole source of vitamines, rats are able to continue their growth to maturity at a rate slightly below that possible on our basal ration when suitably supplemented. Rat 436, after the ration had been supplemented with an additional amount of the fat-soluble vitamine, which had no apparent effect on growth, produced a litter of five young. While normal in weight—they weighed 25 gm.—they failed to be nourished by the mother rat and soon succumbed. An individual failure of this kind has, however, no special significance as regards reproduction.

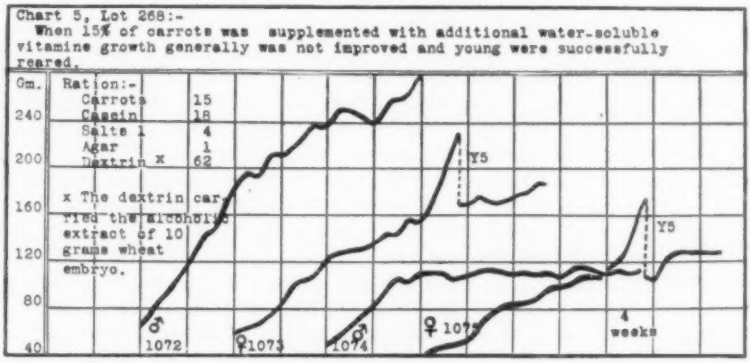


Chart 5. 15 per cent of carrots when supplemented with an additional amount of the water-soluble vitamine did not, with one exception, induce any better growth than when not so supplemented (Chart 3). Rats 1074 and 1075 were decidedly stunted. They gave the impression of being shorter in body length than their weight would normally call for. We have never seen this effect occur in rats where normal growth was disturbed by a lack of vitamines in the diet, but it is a common occurrence where stunting has resulted from digestive disturbances. That the poor growth performance of these animals was not due to a vitamine deficiency is distinctly indicated by the fact that Rats 1073 and 1075 reared five out

of the ten young that they produced. In our experience, the quantity of vitamines required by the rat for normal milk production is considerably greater than that necessary for normal growth. The young reared were, however, far from being normal as they did not attain an average weight of 45 gm. until they were 55 days old; normally in our stock this weight is attained in less than half that time. When weaned, some of the young were very much bloated which gave us our first evidence that the analysis, attributing the cause of the small size of Rats 1074 and 1075 to digestive disturbances rather than to a vitamine deficiency, was probably correct.

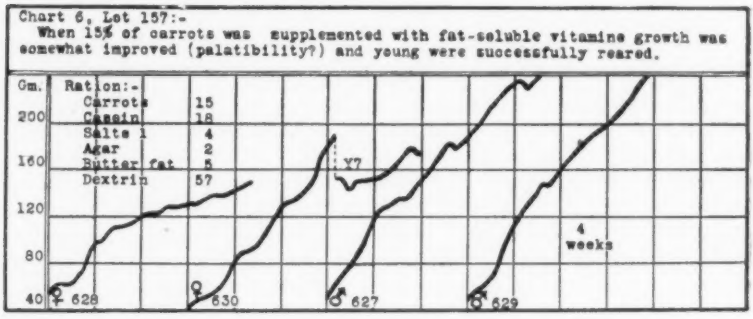


Chart 6. Lot 157 offers additional evidence to that already brought out in Lot 268, Chart 5, that 15 per cent of carrots introduces enough water-soluble vitamine into the diet for normal growth requirements and even for the rearing of some young. In fact there must have been a considerable excess for growth otherwise young could not have been reared as conditions in the tract could not be considered normal. To a certain extent, growth in this lot is better than in Lot 268 where butter fat was not added and water-soluble vitamine was added instead, but it is not pronounced enough to be given special significance.

As in Lot 268 young were reared although only two out of the seven and that at approximately the same subnormal rate, the two together weighing only 111 gm. when 56 days old. In the light of this it is certain that the retarded development of the young was not caused by an insufficiency of the fat-soluble vitamine. Furthermore, in none of the animals was there ever any indication of xerophthalmia.

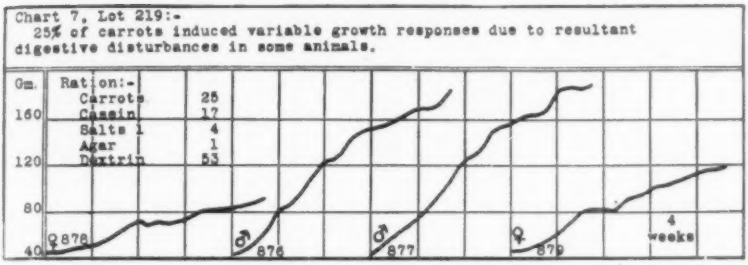


CHART 7. As already indicated by the results pictured in Charts 5 and 6 a high content of carrots in the ration is liable to cause digestive disturbances and as a result of difference in the power of resistance to these disturbances there is considerable variation in the growth performance of different animals. When the amount of carrots is increased to 25 per cent these differences become still more marked as the disturbances are aggravated so that tympanites was regularly observed.

It is to be noted that the results obtained on these carrot rations even when carrying but a low percentage of carrots were never entirely satisfactory from the standpoint of normal nutrition. When the carrot content was too low, the vitamine deficiency would become evident, and if too high or even just high enough for the proper vitamine relations, the large amount of fermentable but indigestible carbohydrate introduced into the ration would cause digestive disturbances and the resultant marked interference with normal growth. For this reason it was not considered advisable to introduce more than 25 per cent of carrots into the ration. In one case where as much as 60 per cent of carrots was fed diarrhea and tympanites always resulted and rendered the results difficult of interpretation.

By adopting such a low level of carrots in the diet that the disturbing effects were minimized, we were able to demonstrate that carrots are remarkably rich in the fat-soluble vitamine. In spite of growth not being entirely satisfactory, this is the only conclusion that can be arrived at in view of our findings that on as low a level as 15 per cent of carrots as the sole source of the fat-soluble vitamine female rats are able to raise their young without any indications of a deficiency.

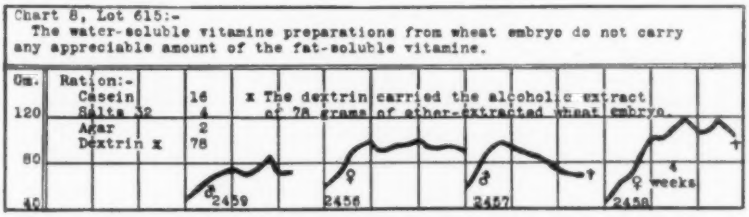


CHART 8. In experiments where additional water-soluble vitamine was added the amount of fat-soluble vitamine added with it was extremely

small as brought out by the growth performance of Rats 2456 and 2459 where they received almost three times as much of the preparation as any of the other experimental groups. In this group Rat 2458 died from xerophthalmia and Rats 2456 and 2459 both had inflamed eyes at the time of their death. It is not necessary therefore to make any reservations in the conclusions so far drawn with respect to the amounts of fat-soluble vitamine present in those rations where water-soluble vitamine had also been added.

From our data and from what is known of their antiscorbutic properties it appears that the practice of feeding carrots in lieu of green materials in vogue by many small animal breeders is dietetically justified from the vitamine standpoint alone. It is also possible that herein lies experimental justification for the use of carrot juice as an adjuvant to the boiled milk diet of children.

Fat-Soluble Vitamine in Swedes or Rutabagas.

The roots used in these experiments were of a pale yellow color and of excellent quality. They were washed, pulped, and then dried in an air current at room temperature and subsequently over calcium chloride to facilitate their comminution in our milling apparatus. When fed, exposure to the air and light had bleached them until they were almost void of all yellow color.

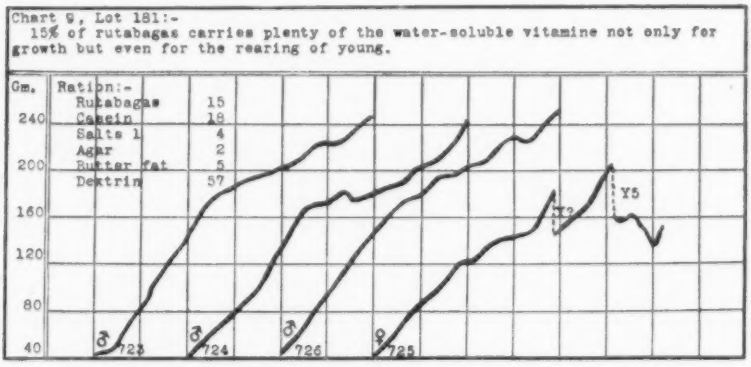


CHART 9. As it is always necessary to insure a sufficiency of all the dietary requirements outside of the unknown which it is desired to determine, a diet containing 15 per cent of rutabagas suitably supplemented with casein and salts was also supplemented with a sufficiency of the fat-soluble vitamine as found in butter fat to allow determination of the

amount of water-soluble vitamine present. As is seen in the chart, growth was practically normal, furthermore Rat 725 reared her second litter of young to weaning. Their growth was not entirely normal, yet they averaged 42 gm. in weight when 33 days old which speaks well for the nutritive sufficiency of the ration. All the rats were in good condition at the time of termination of the experiment, 24 weeks after its inauguration.

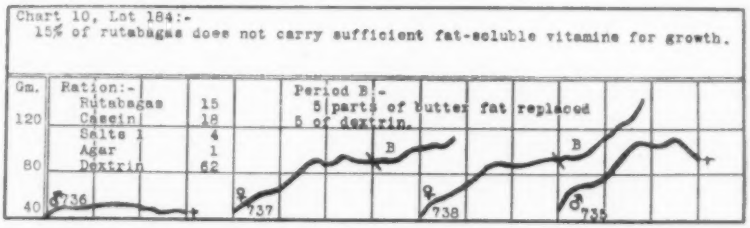


Chart 10. With a sufficiency of the water-soluble vitamine in 15 per cent of rutabagas established (Chart 9), it was comparatively an easy matter to determine its relative fat-soluble vitamine content. Unlike carrots (Chart 4) 15 per cent of rutabagas as the sole source of the fat-soluble vitamine leads to early indications of its deficiency. Rat 735 became afflicted with xerophthalmia which ultimately, after 12 weeks, was the immediate cause of its death. Rat 737 likewise contracted xerophthalmia, but on the addition of fat-soluble vitamine in the form of butter fat, the inflammation disappeared rapidly so that no further signs of it were noted during the last 6 weeks of the experiment; simultaneously it more than maintained its weight and appeared to be in fair nutritive condition. Rat 738, while it had not contracted xerophthalmia, improved remarkably in appearance after the addition of butter fat to its ration. It was short, however, and gave the impression of having been stunted not by a nutritive deficiency, but by digestive disturbances.

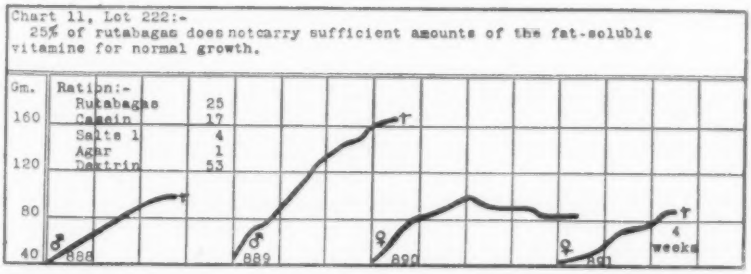


Chart 11. When the amount of rutabagas was increased to 25 per cent of the ration the demands for fat-soluble vitamine still remained unsatisfied. This cannot be inferred from the observed failure of normal

growth to result because digestive disturbances attributed to the hemicellulose variety of carbohydrates complicated the situation just as in the case of the carrots. Rat 890 was the only rat that contracted xerophthalmia. Rat 889 was bloated almost continually and ultimately died from this condition. We are inclined to believe that the more pronounced tympanites was due to the greater amount of food consumed by this animal. If it consumed more food, this would easily explain its more pronounced growth as thereby its vitamine intake was satisfied. In such instances as this individual records of food consumption would facilitate the interpretation of data. In most instances, however, accurate conclusions can be arrived at by the law of averages. In the case of Rats 888 and 891 no tympanites was observed and at the time of their death their eyes were perfectly normal.

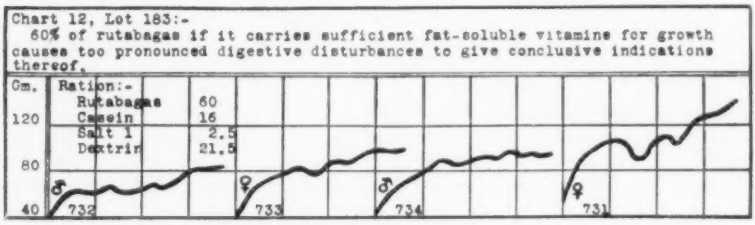


CHART 12. Whether sufficient amounts of fat-soluble vitamine to satisfy the requirements for growth in the rat can be introduced into the ration with dried rutabagas is difficult of determination. Whenever large amounts of the root are incorporated in the diet digestive disturbance which appeared even when only 25 per cent of the root was fed became so aggravated that normal growth was impossible. At a level of 60 per cent it is noteworthy that while the diet was rendered distinctly unfavorable for growth on account of the persistent tympanites and occasional diarrhea no xerophthalmia was observed in any of the animals during the 15 weeks of the experimental period. If the rutabagas contained none of the fat-soluble vitamine it was to be expected that xerophthalmia would have resulted in the presence of these digestive disturbances and over such a protracted period. But final conclusions cannot be arrived at with these limited data. There is no question, however, that if rutabagas contain any demonstrable amounts of the fat-soluble vitamine they are not to be considered comparable to those found in carrots.

Fat-Soluble Vitamine In Dasheens.[17]

The dasheens, both corms and roots of medium size and free from foreign material, were sliced without peeling and then dried

[17] The common name of dasheen is taro, and the scientific name *Caladium colocasia*.

at room temperature in an air current. In this condition they could be readily ground to a fine powder and incorporated in the ration. No difficulty in securing consumption was observed.

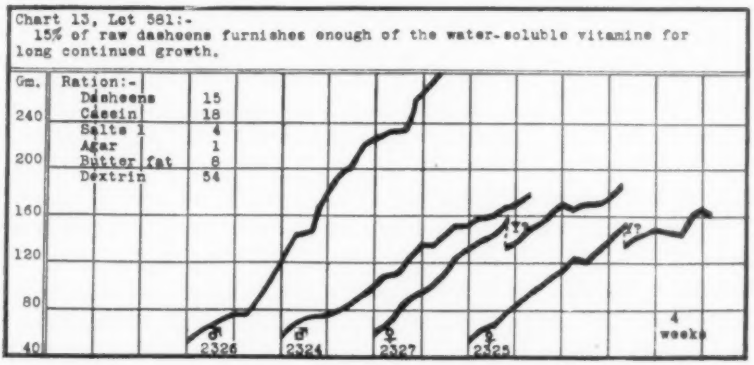


Chart 13. From the performance of the animals in this lot there remains no question but what dasheens are abundantly supplied with the water-soluble vitamine to support long continued growth of the rat. It is true that the observed growth was not what could be considered normal, but nevertheless for our purposes of comparison the results must be considered very satisfactory especially in view of the fact that no rations which can be considered synthetic in any sense of the word are entirely satisfactory even for growth and certainly not for reproduction and rearing of the young.

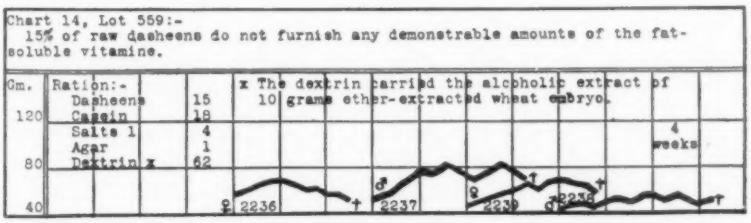


Chart 14. In a ration provided with a liberal excess of the water-soluble vitamine and all other nutritive factors, outside of the fat-soluble vitamine necessary for growth, provided for, 15 per cent of dasheens does not take care of this deficiency to allow even the minimum amount of growth. Rat 2339 became blinded by the resultant xerophthalmia but the other rats in their miserable condition became infested with lice, making it difficult to ascertain definitely before their death—which followed shortly—whether or not the inflamed condition of the eyes was due to the fat-soluble vitamine deficiency or to the irritation. Under the conditions,

the consistent failure of growth together with the occurrence of one clean-cut case of xerophthalmia speaks decisively for a fat-soluble vitamine deficiency in this ration.

In several instances attempts were made to feed a higher percentage of dasheens. This resulted in complete failure, but for reasons which had hitherto not been appreciated. When raw dasheens are fed in liberal amounts it becomes evident that they are very difficultly digestible which fact became especially evident when the amount was increased to as high a content as 80 per cent of the rations. Even though the rations were complete in every way the indigestibility of raw dasheen starch prevented the rats from even maintaining themselves. Large quantities of the feed were devoured at times and bulky fecal residues were eliminated due to the excretion of undigested starch as indicated by the iodine potassium iodide reaction. Autopsy of a number of animals indicated that the abdominal distention which had at first been taken as due to gaseous fermentation was in reality due to an abnormal enlargement of the cecum and large intestine in accommodation to the accumulation of the undigested food.

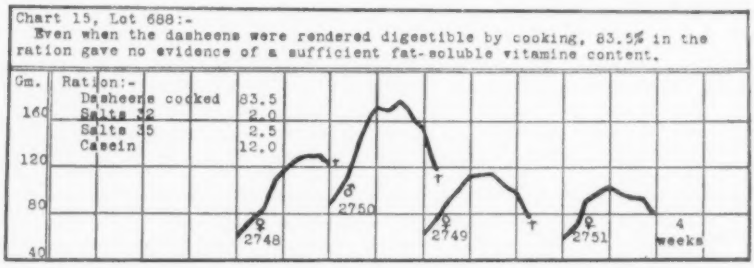


Chart 15. As the indigestibility of the starch precluded the possibility of demonstrating the low fat-soluble vitamine content of dasheens it became necessary to cook them. When cooked, difficulty in the digestibility of the starch was no longer observed, but neither was there made evident the presence of a fat-soluble vitamine content. Viewed in the light of results obtained later we are thoroughly convinced that the data are representative of what would have been obtained on the raw dasheens if results had not been obscured by their indigestibility. It is probable that in spite of their indigestibility when 15 per cent of the raw dasheens was fed if they had contained any demonstrable amount of the vitamine it would still have been shown by the growth responses as the demonstration of the presence of the water-soluble vitamine was not

interfered with by this condition (Chart 13). Furthermore the dasheens fed were cooked at a low temperature (5 pounds steam pressure for 20 minutes) and were then dried at room temperature in an air current. From what we now know of the stability of the fat-soluble vitamine in plant materials the amount destroyed must have been very small if at all demonstrable by feeding experiments. If the fat-soluble vitamine occurs at all in the dasheens it should here have become evident.

Fat-Soluble Vitamine in Red Beets.

The red beets of medium size were scrubbed thoroughly, then sliced, and dried in an air current at room temperature. Under these conditions and even when kept over anhydrous calcium chloride for some weeks they did not dry enough to make it possible to grind them. Only by drying them in a thin layer for 20 minutes at 90° did they become brittle enough to grind and later pulverize to an impalpable powder in a ball mill.

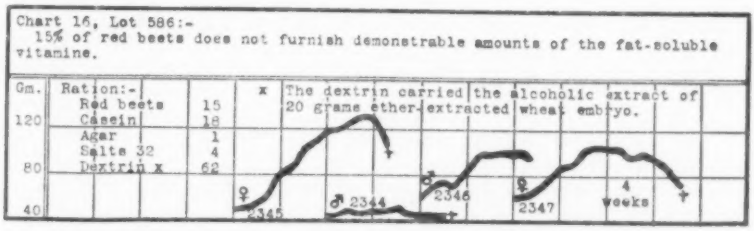


Chart 16. In a ration complete in its content of the dietary essentials requisite for fairly good growth with the exception of the fat-soluble vitamine 15 per cent of red beets does not furnish enough of this vitamine. None of the rats grew at the normal rate for even a limited time and one, Rat 2344, remained absolutely stationary in body-weight. Rats 2345 and 2347 developed bad cases of xerophthalmia which persisted to the time of their death. Rat 2344 died without any specific symptoms of vitamine deficiency.

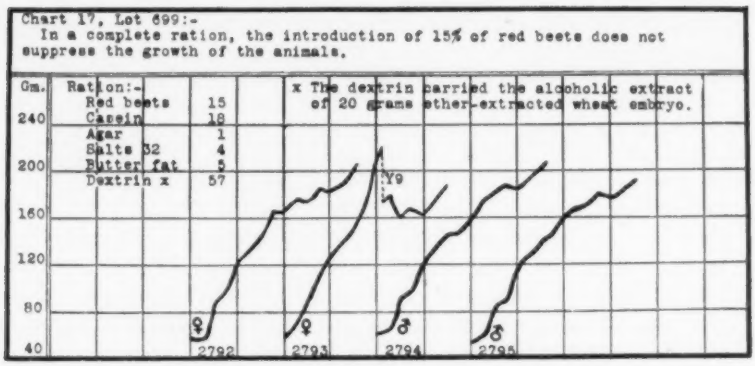


CHART 17. Proof that the failure of growth in Lot 586, Chart 16, was not due to any unfavorable factors in the diet introduced with the 15 per cent of red beets is brought out by this lot of animals. When added to a complete diet no prejudicial effects were observed. It must be remembered, however, that this evidence is not absolute as in a satisfactory ration factors slightly detrimental in action would be obscured by the greater powers of resistance of the animal under these conditions. It can be safely concluded that red beets are very low in or practically free from the fat-soluble vitamine.

Fat-Soluble Vitamine in Parsnips.

The roots were sliced and then dried at room temperature in an air current. While fairly dry, they were sufficiently hydroscopic to remain tough until dried over calcium chloride; after that, they could be readily pulverized. The comminuted preparation was of a faint but clear yellow color which appeared to have greater permanency than the yellow of the rutabagas which as already noted bleached out rapidly.

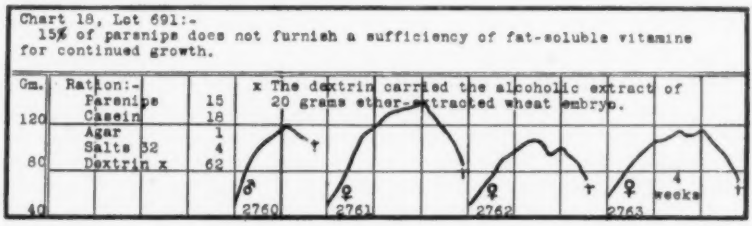


CHART 18. In two experimental lots of which the record of only one, *viz.* 691, which showed the most growth is here reproduced no evi-

dence of a sufficiency of the fat-soluble vitamine for growth was obtained. Rat 2760 had a severe attack of indigestion resulting in diarrhea which probably was indicative of an unfavorable character of the carbohydrate in the root as demonstrated in the case of the carrot and rutabaga. Rats 2761, 2762, and 2763 all had inflamed eyes at the time of their death, but as the eyes did not become purulent before death we are not positive whether we were dealing with xerophthalmia due to fat-soluble vitamine deficiency or not. In view of the ultimate failure in the growth performance of the rats in two lots, though the ration was initially apparently relished by the animals, together with the incipient eye inflammations we believe we are justified in concluding that parsnips are poor in their fat-soluble vitamine content. We did not determine whether larger amounts might not have been satisfying in furnishing this vitamine.

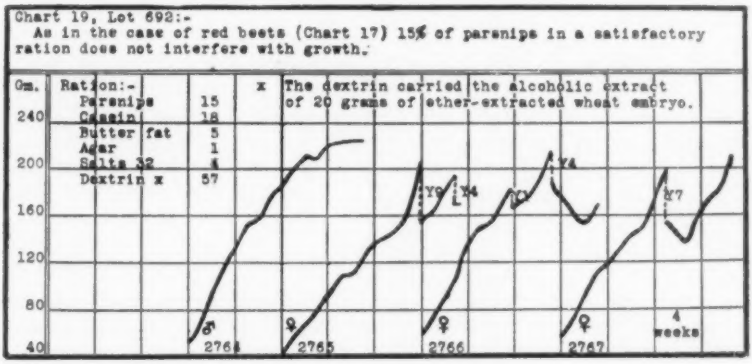


Chart 19. This record makes it positive that failure in Lot 691, Chart 18, was not due to harmful constituents or unpalatable constituents in the parsnips as growth was uniformly satisfactory when the ration was complete. In the 1st week the average consumption was 39 gm. per rat as compared with 40 gm. in Lot 691, in the 2nd week 53 gm. as compared with 57, and in the 3rd week 64 as compared with 64. These records were obtained by means of a special feeding device which it is believed gives as satisfactory data of food consumption as it is possible to secure with rations of the physical characteristics here employed.

Fat-Soluble Vitamine in Potatoes.

The data on the vitamine content of potatoes here reproduced were obtained at various intervals over a considerable period of time and of all the records accumulated, only a few have been presented to indicate the nature of the problem and to present the general trend of the results obtained.

The tubers were bought on the open market and were of medium size. They were washed free from dirt and then sliced without paring. Those fed raw were dried at room temperature while those fed cooked were autoclaved at 15 pounds pressure from 60 to 75 minutes. Generally after cooking they were dried in an oven at 90°C. An exception to this procedure is to be noted, Chart 24, Lot 719, where they were dried in an air current at about 30°C.

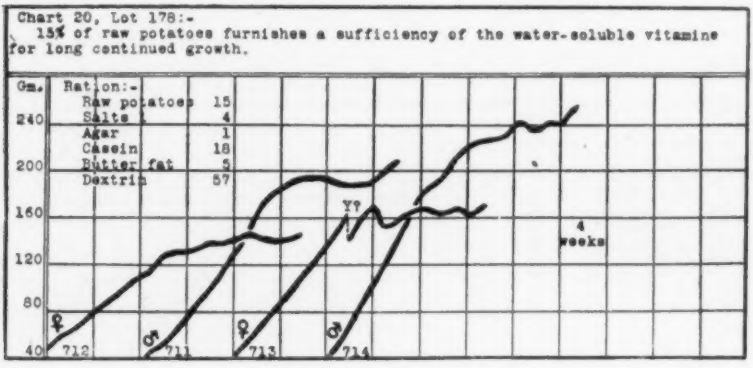


Chart 20. Potatoes furnish us with another instance of the relatively large amounts of water-soluble vitamine present in proportion to the animal's requirements for growth. This does not necessarily mean that the amount present in their diet is generally much larger than the animal requires for its physiological well being—though evidence points in this direction—but it does mean that on a diet of roots the animal is certain to feel the need of other constituents before that of the water-soluble vitamine.

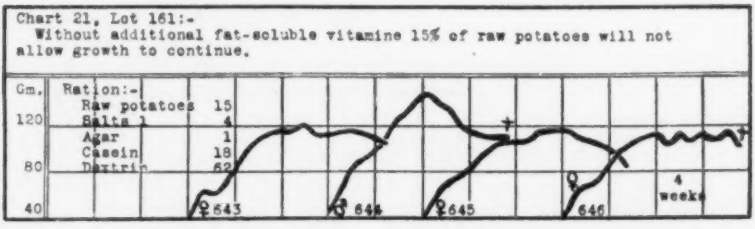


Chart 21. When the ration fed to Lot 178, Chart 20, was not provided with fat-soluble vitamine in addition to that furnished by the 15 per cent of raw potatoes, failure in nutrition results. Here again, then, we have an instance where, relative to the needs of the animal for growth, a

deficiency in the fat-soluble vitamine becomes especially evident. After failure to grow had been decisively indicated the ration was changed to the extent that five parts of dextrin were replaced by five of butter fat. Before the effect of this vitamine addition had become evident the animals died, death being directly due to their poor nutritive condition.

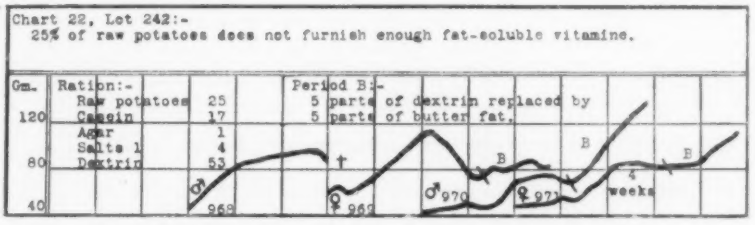


Chart 22. With such amounts of fat-soluble vitamine as are present when 25 per cent of the ration is supplied as raw potatoes, the observed growth performance was less than on 15 per cent (Chart 21, Lot 161). This is due to the fact, as was discovered later when large amounts of raw potatoes were fed, that raw potato starch is digested with great difficulty by the rat. Therefore, as the amount of potato in the ration was increased, such improvement as would result from any additional amount of the fat-soluble vitamine present was masked by the unfavorable effect of the decrease in the amount of available energy. There remains, however, absolutely no doubt that the failure of continued maintenance on this ration was primarily caused by a lack of the fat-soluble vitamine as Rats 968, 969, and 971 all contracted xerophthalmia while on the ration.

In the light of what has been said the improvement in the condition of the rats when butter fat was added as indicated on the chart should not be accepted without reserve as being due to the fat-soluble vitamine thereby added. In part the beneficial results are to be attributed also to the increased intake in the required amount of energy of which the animal was otherwise deprived by the indigestibility of the starch.

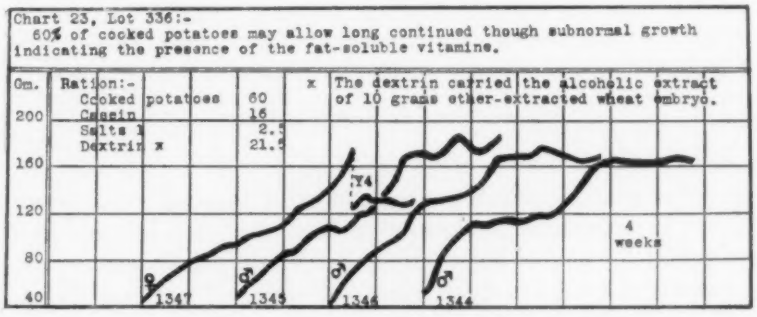


Chart 23. As when even low percentages of raw potatoes were present in the ration there was marked evidence of their indigestibility it

obviously was an impossibility to attempt to demonstrate the presence of the fat-soluble vitamine in such potatoes. Much as we felt disinclined, we were forced to cook them to render them digestible. On potatoes so treated, much to our surprise, the rats continued to grow for a considerable period of time and later maintained themselves for a period of 23 weeks with but 60 per cent of potatoes in the ration as the source of the fat-soluble vitamine. At the end of this time none of the rats gave signs of impending nutritive failure.

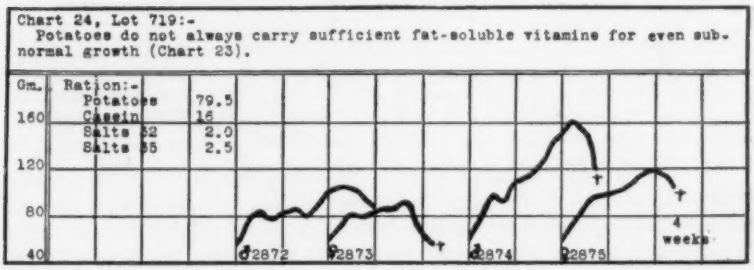


CHART 24. That much reliance is not to be placed on the general occurrence of the fat-soluble vitamine in the potato is brought out in this experiment. The potatoes used were from a different lot, but were prepared for feeding as those used for Lot 330 with the exception that later they were air-dried so that the treatment to which they were subjected was even milder than that used in the other instance and therefore more of the original vitamine content should have been preserved. The general miserable condition of these animals testified to the fact that even with 19 per cent more of potatoes in the ration as compared with Lot 336 there was far less fat-soluble vitamine demonstrable. We are forced to the conclusion that there is considerable variation in the fat-soluble vitamine content of potatoes, a fact the importance of which we did not appreciate until similar observations of variations were obtained with other plant materials. In general it is believed safe to infer that potatoes may contain enough of the fat-soluble vitamine for normal growth, but generally they can be considered poor in their content of this dietary essential.

Fat-Soluble Vitamine in Mangels.

The mangels used in these experiments were a variety known as sugar mangels which are considered of superior value on account of their higher sugar content to the ordinary mangels used as fed by the animal husbandman and so extensively used by the Germans for human food during the time of food scarcity in 1917. They were washed, pulped on a power beet rasp, and then

dried in an air current at room temperature. In air-dried condition they were readily pulverized and incorporated in the ration.

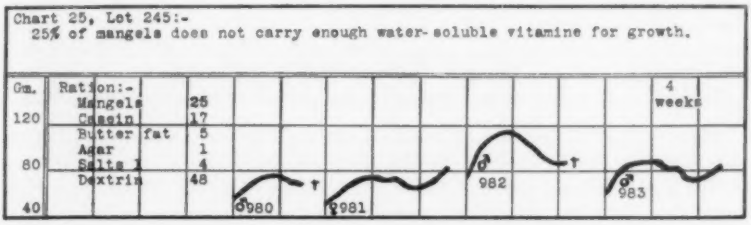


CHART 25. Unlike carrots, rutabagas, dasheens, potatoes, beets, and parsnips 25 per cent of mangels does not furnish the rat with the required amount of water-soluble vitamine. In an otherwise satisfactory ration the lack of water-soluble vitamine was indicated by the failure of the animals to grow and in case of Rat 980 by an attack of convulsions characteristic of polyneuritis which terminated fatally.

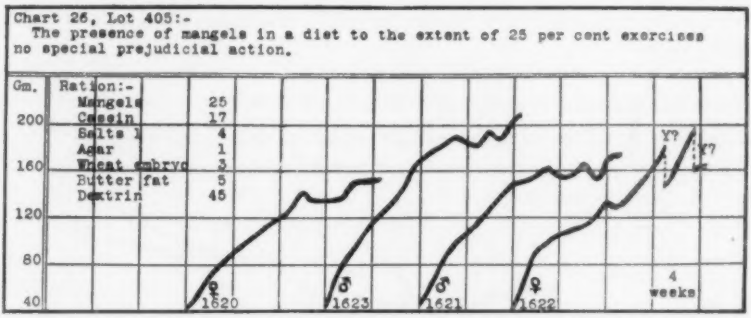


CHART 26. The growth performance of this group of rats on a ration different from that of Lot 245, Chart 25, only in that three parts of dextrin were replaced by ether-extracted wheat embryo—well known to be rich in the water-soluble vitamine—demonstrates that no mistake was made in attributing the cause of failure in Lot 245 to a deficiency of the water-soluble vitamine.

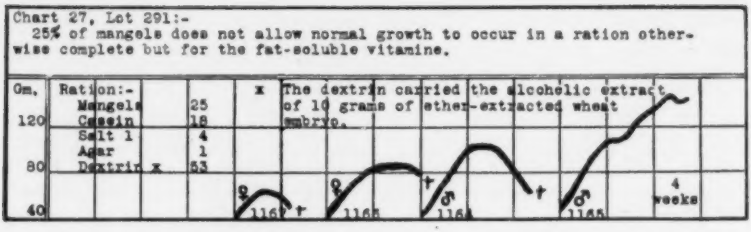


CHART 27. Besides their poverty in the water-soluble vitamine mangels are also deficient in the fat-soluble vitamine as indicated by the results of this experimental group. Rats 1164, 1166, and 1167 died when in a very miserable condition due to a poor nutritive state of the skin. The general condition of Rat 1165 was no better than the others, but it maintained its weight better for the time that the experiment was continued.

Fat-Soluble Vitamine in Sugar Beets.

Sugar beets prepared for feeding by pulping and then drying in an air current at room temperature and finally over calcium chloride indicated the same general vitamine relations as the mangels.

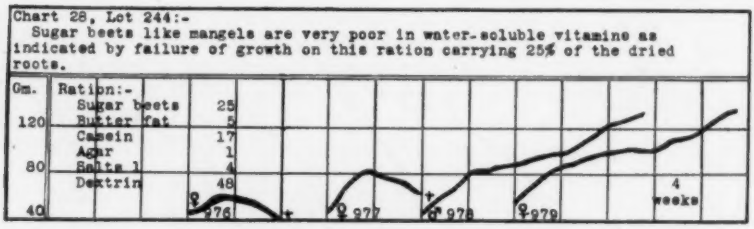


CHART 28. Rats 976 and 977 failed to live longer than 8 weeks on a ration dependent for the water-soluble vitamine on the amount introduced with 25 per cent of sugar beets. Their death was directly due to this deficiency as both died after having shown severe convulsive symptoms common in attacks of polyneuritis. Rats 978 and 979 did not succumb to this deficiency; neither was their growth satisfactory. Such variations are merely instances of certain individuals being better able to withstand an unfavorable environment.

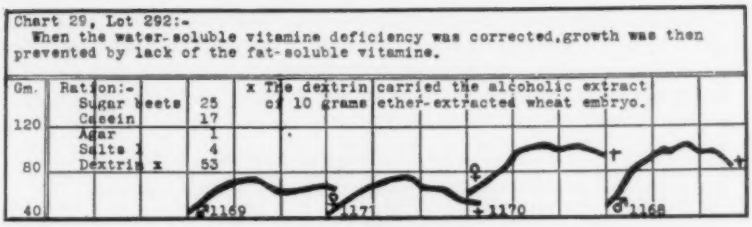


CHART 29. The evidence for a fat-soluble vitamine deficiency in a ration dependent for this dietary essential on 25 per cent of sugar beets was so pronounced that no further experiments to determine this were inaugurated; Rat 1169 was totally blinded by the xerophthalmia, Rat 1170 contracted xerophthalmia in one of its eyes before death, and Rat 1171 in both eyes. It was only in the case of Rat 1168 that no symptoms of vitamine deficiency outside of failure to grow were observed.

Fat-Soluble Vitamine in Yellow Sweet Potatoes.

Next to the Irish potato there is no tuber or root crop which enters into the make-up of the human diet to such a large extent as the yellow sweet potato, especially when it is in season as its poor keeping qualities more than anything else has prevented its continued use. The favor with which it is received in the American home makes a comparison of its dietary properties with the Irish potato important.

The potatoes used for most of the following investigations were peeled potatoes that had been dried at a temperature of 50–60°C. In three instances air-dried potatoes were fed for purposes of comparison.

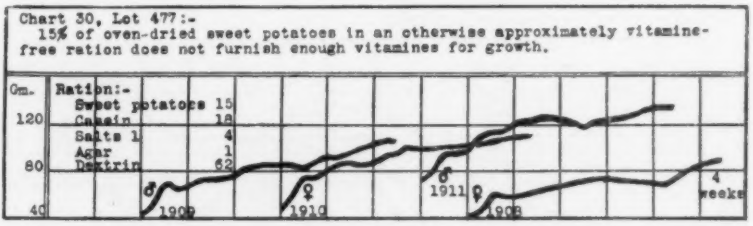


CHART 30. 15 per cent of sweet potatoes as a source of vitamines in the diet of young growing rats led to a very peculiar rate of increase in weight. Growth at a very slow rate was continued for 22 weeks during which there were no signs of polyneuritis or xerophthalmia. The form of the curve, together with the general body condition of the animal—

recorded as good with the exception of Rat 1908 which was designated fair—is most suggestive of a water-soluble vitamine deficiency. Evidently there was just enough vitamine present to maintain the animals. It remained to establish whether or not at this level of sweet potato feeding a deficiency of the fat-soluble vitamine did occur.

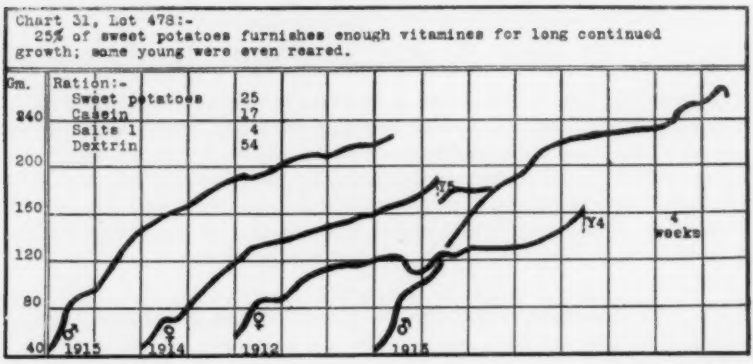


Chart 31. That growth on sweet potato vitamines becomes possible is shown when such an amount of sweet potatoes is introduced into the ration that it makes up 25 per cent of the total. This experimental group indicates that in the previous lot (Lot 477, Chart 30) failure of growth was not primarily due to any unsatisfactory factors introduced into the ration with the potatoes and that therefore it must have been due to a lack of vitamine. The performance of this group of animals is rather remarkable, not only in the growth responses, but also in the fact that one animal, Rat 1914, raised young. She raised three out of a litter of five to an average weight of 37 gm. in 5 weeks. Though undersized they were in good condition.

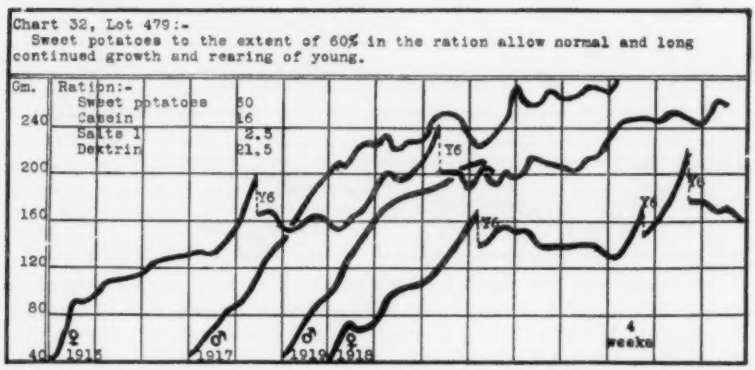


Chart 32. With the per cent of sweet potatoes increased to 60 the rate of growth was more nearly what could be considered normal for the

rats of our colony. But as noted on the chart there occurred numerous variations in the weights of the individuals which are indicative of digestive disturbances as often indicated by the bloat observed from time to time. That the vitamine requirement for growth must have been generously satisfied must be inferred from the record of reproduction. Rat 1916 raised five out of a litter of six young to an average weight of 48 gm. in 5 weeks and 4 days, and all of six young from a second litter to an average weight of 42 gm. in 5 weeks (see Plate 1). The other female, Rat 1918, raised two young out of her first litter of six to an average weight of 38 gm. in 7 weeks and all of eight young in her third litter to an average weight of 61 gm. in 7 weeks and 4 days. The second litter was not raised for reasons unknown as they disappeared from the cage a few days after birth, evidently having been consumed by the mother. There certainly is nothing to indicate that the sweet potato cannot be an important source of vitamine in the diet. None of these animals showed any signs of premature senility at 10 months which is further evidence of the satisfactoriness of the diet.

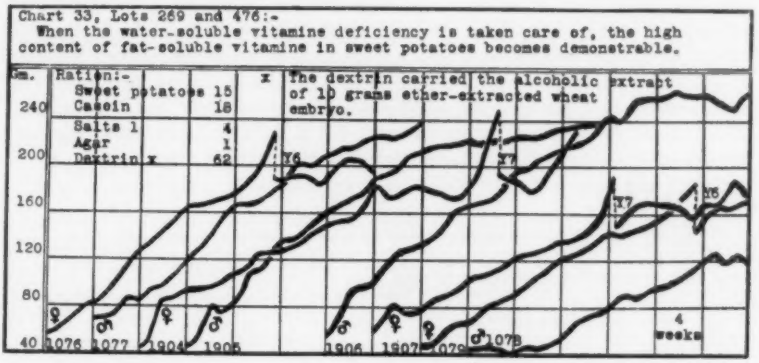


Chart 33. The experimental results of two separate feeding trials indicate that the primary vitamine deficiency in our sweet potato material was the water-soluble vitamine deficiency. When this was corrected by the addition of an alcoholic extract of ether-extracted wheat embryo fairly good growth, together with rearing of the young, became possible. Neither the growth of the original lots nor the growth of the offspring was normal, but nevertheless the performance was remarkable as growth was long continued. Rat 1077 in the 19th week developed an edematous eye and Rat 1078 an inflamed eye. These abnormal conditions were temporary and later entirely disappeared. They were diagnosed as not being caused by a fat-soluble vitamine deficiency and, as the attacks were not of long duration and were not accompanied by great losses in weight, they were taken as being due to trauma. This later appeared entirely justifiable in view of the success in reproduction. Rat 1076 raised four young to an average weight of 59 gm. in 9 weeks, and Rats

1904 and 1907 five young to an average weight of 44 gm. in 7 weeks and 4 days. These must not be taken as instances of satisfactory rearing of young, as their rate of development was only approximately one-half of what it should be; it was remarkable that young were raised at all.

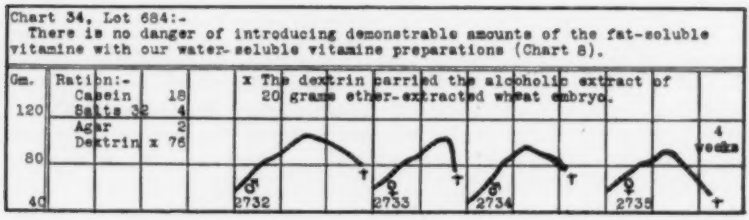


Chart 34. While we had demonstrated once before (Chart 8) that our water-soluble vitamine preparation contains little if any fat-soluble vitamine, the demonstration, on account of the importance of the data just discussed, was here repeated. On a ration containing the alcoholic extract of 20 gm. of ether-extracted wheat embryo as the sole possible source of the fat-soluble vitamine all the rats succumbed within 3 months and all had infected eyes, although Rat 2732 owed its death directly to an abscess on its jaw and Rat 2733 to a pulmonary infection. The results prove that the growth in Lots 269 and 476, Chart 33, could not possibly have been due to the high fat-soluble vitamine content of the water-soluble vitamine extract.

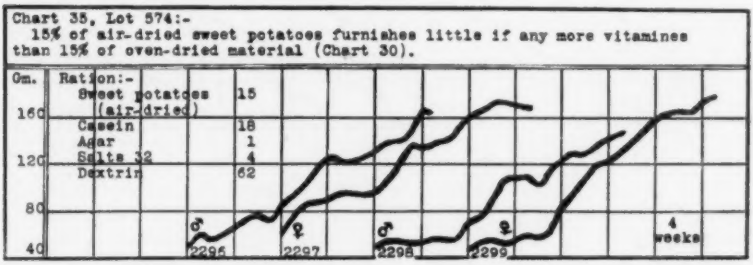


Chart 35. In spite of the data on the stability of the water-soluble vitamine now at hand in the literature, it cannot be taken for granted that the vitamine is left intact in the process of drying where temperatures higher than room temperature are employed. The sweet potatoes used in this and the following two lots were obtained on the local market. They were peeled, then sliced, and dried in an air current at room temperature. On them, at a level of 15 per cent as the source of vitamines, growth was slow and indicated a lack of vitamines as in Lot 477, Chart 30, of the oven-dried material.

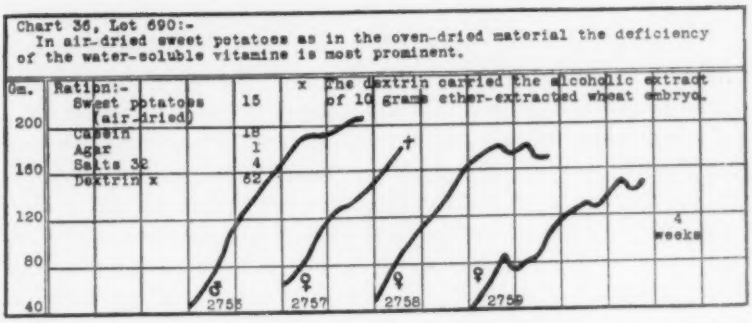


CHART 36. 15 per cent of the air-dried sweet potatoes when fortified with water-soluble vitamine enables more rapid growth to occur than without this addition. The experiment has not yet been concluded, but the indications are that the order of magnitude of the vitamine deficiencies of a vitamine-free ration having its vitamine introduced with small percentages of air-dried sweet potatoes are of the same character as those of the oven-dried material.

CONCLUSION.

From the data of the present series of investigations it is seen that the vitamine relations in roots may be of a widely differing character. With 15 per cent of the diet made up of roots as the source of the fat-soluble vitamine we have in the case of the yellow sweet potato and carrot normal growth and even rearing of the young made possible, but in the case of the rutabaga, dasheen, red beet, parsnip, potato, mangel, and sugar beet complete failure resulted. In fact, in some instances failure at such higher levels as were dried—25 per cent in the case of the mangel and sugar beet and 83 per cent in case of the dasheen—was also observed.

Likewise, from the data here presented—which are representative of a large number of experiments—the conclusion seems warranted that tubers and roots are not necessarily to be classed with food materials grossly deficient in their fat-soluble vitamine content. While in some instances it is true that there is little or no fat-soluble vitamine demonstrable, in other instances there is enough present to warrant their classification with respect to their content of this dietary essential with leafy materials rather than with our cereal grains such as maize, wheat, barley, or oats.

It must not be taken for accepted that absolute comparisons of the amounts of the vitamine to be found in different plant materials are possible as our knowledge of the occurrence of the vitamine is too limited. At any rate it is probable that not until we know something of the function of this vitamine in the plant kingdom, or at least know something of its association with specific principles or physiological processes that general statements on the basis of such limited data as here presented will be warranted. The danger of drawing conclusions from limited data is brought out graphically in the case of the experiments carried out on potatoes. In only one out of two instances was the presence of the fat-soluble vitamine demonstrated when different samples were fed at high levels. We believe that this is only one of numerous instances of variation in the natural occurrence of vitamine which later may be easily understood as their physiological rôle is appreciated. We believe, however, that our general conclusions in regard to the especially high fat-soluble vitamine content of carrots and yellow sweet potatoes as compared with red beets, parsnips, rutabagas, sugar beets, potatoes, mangels, and dasheens will not need qualification.

The water-soluble vitamine relations as brought out in our experiments are also worthy of some comment. In the case of the carrot, rutabaga, and dasheen 15 per cent of the material furnished enough of this vitamine for growth. Of sweet potatoes a somewhat larger amount was necessary, but of the sugar beet and mangel, even as much as 25 per cent of the ration gave no evidence of furnishing this compound.

In comparing the relative amounts of fat-soluble vitamine and water-soluble vitamine occurring in the various materials studied it is noteworthy that there is no evident relation between them as measured by the amount required to enable the rat to grow. While in dasheens no fat-soluble vitamine could be demonstrated no matter what the amount fed—though 15 per cent furnished enough water-soluble vitamine—in carrots on the other hand enough of both the fat- and water-soluble vitamine was furnished by an amount equivalent to 15 per cent of the ration. Again, in mangels and sugar beets both vitamines were present in such small amounts, if at all, that they could not be demonstrated when fed at a 25 per cent level. From the standpoint of plant

physiology it is difficult to surmise just what these relations signify, but most certainly it does not appear justifiable to associate generally great physiological activity with an abundance of vitamine.

Acknowledgments and thanks are due to Dr. J. S. Caldwell, Dr. R. A. Young, and Dr. P. H. Dorsett of the Bureau of Plant Industry, Washington, D. C., for cooperation in the experiments to the extent of supplying the authors with dasheens and sweet potatoes suitable for these experiments.

EXPLANATION OF PLATE 1.

FIG. 1. Rat 1916 was raised on a ration which derived its entire content of vitamine, both fat- and water-soluble, from sweet potatoes which made up 60 per cent of it (Chart 32). Started on the ration at 45 gm. September 9, 1918, she weighed 215 gm. June 9, 1919. As seen from the picture she was in excellent nutritive condition just after she had raised the second litter of young.

FIG. 2. Two out of the litter of six young all of which were raised by Rat 1916 on the 60 per cent sweet potato ration as the source of vitamines. When photographed at the age of 5 weeks and 4 days they averaged 53 gm. in weight. Though normally on our stock ration they would have weighed this 12 days sooner, their performance on this restricted ration is none the less remarkable.

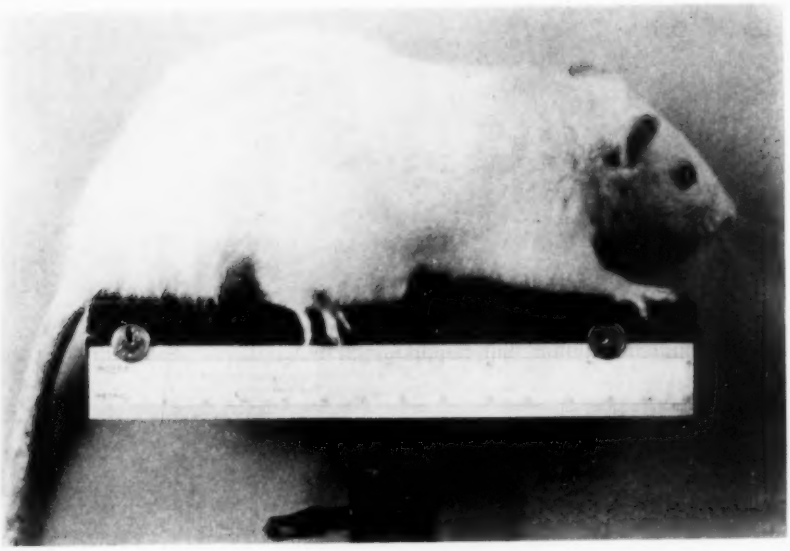


FIG. 1.

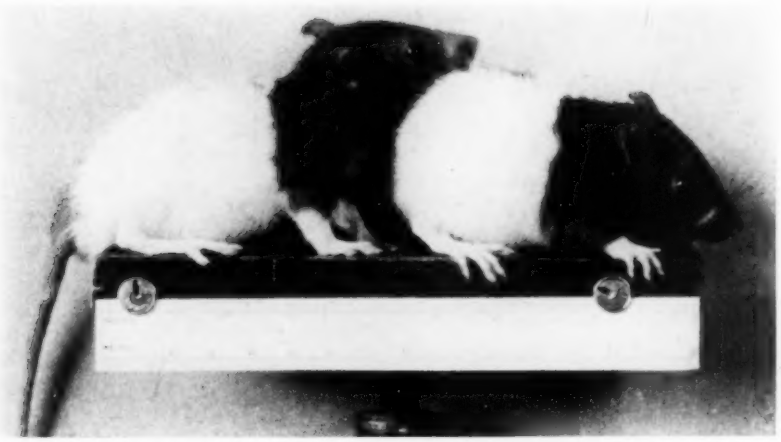


FIG. 2.

CHEMOTHERAPEUTIC STUDIES ON ORGANIC COMPOUNDS CONTAINING MERCURY AND ARSENIC.*

By GEORGE W. RAIZISS, JOHN A. KOLMER, AND JOSEPH L. GAVRON.

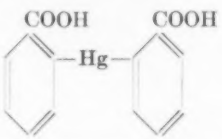
(From the Dermatological Research Laboratories, Philadelphia.)

(Received for publication, October 29, 1919.)

The valuable properties of mercury compounds in the treatment of spirochete infections are well known. Prior to the discovery of the dihydrochloride of diaminodioxyarsenobenzene, widely known as salvarsan, mercury compounds were practically the only remedies known for syphilis. While the modern therapy of syphilis is primarily based on the use of salvarsan, metallic mercury, and its inorganic and organic derivatives remain important aids in combating the infection. The elaboration of a mercury compound possessing more powerful spirochetocidal properties, also less toxicity for the body than any of the known mercury compounds, is of great importance. Attempts to synthesize such a compound were numerous (1). As yet none of the new organic mercury compounds has shown superior properties in the treatment of syphilis which would make it more useful than any of the old mercury preparations employed for years by physicians.

The researches of Dimroth (2), Pesci (3), Schrauth and Schoeller (4), and others suggest the advisability of dividing organic mercury compounds in three classes.

To one class belong the full complex compounds in which mercury is attached to carbon of two organic compounds as for instance in Pesci's mercury dibenzoic acid:



* This work was made possible by funds accruing from the dispensing of arsphenamine.

Fischer (5) and also Müller, Schoeller, and Schrauth (6) demonstrated that mercury compounds of the foregoing type are less toxic, but at the same time therapeutically less active than any other class. The reason probably lies in the fact that mercury is so firmly bound to the carbon atoms as to be almost entirely deprived of its metallic properties. This view is supported by observation that mercury cannot be split off even when these compounds are treated with hydrogen sulfide in boiling hot solution.

To the second group comprising the pseudocomplex compounds, belong mercury salts of organic acids, also compounds where hydrogen of a hydroxyl or amino group is replaced by the metal. They are characterized by the ease with which mercury is split off by the action of diluted solutions of caustic soda or hydrogen sulfide in the cold.

The last and most important group includes the so called half complex mercury compounds in which one valence of the metal is attached to the carbon atom of the benzene ring and the other to an inorganic group such as hydroxyl, halogen, or an acetic acid radical. This group is characterized by a comparative stability.

The firmness with which mercury is bound to the organic compound is decidedly greater in half complex compounds than in pseudocomplex. It varies with different compounds and is probably dependent upon the presence of various other groups in the benzene ring.

It is possible that there is relationship between the firmness of the position of mercury in the organic compound and the therapeutic or germicidal effect. Such a relationship, however, can not yet be established.

Two of the authors, for the past 5 years, have been engaged in a series of chemotherapeutic studies with Dr. Jay Frank Schamberg. Organic mercury compounds were to a considerable extent the subject of these investigations. The half complex compounds were thought to be the most suitable for chemotherapeutic study and a considerable number were prepared. Their chemical and biological properties will be the subject of this and subsequent papers. In this communication we intend to describe a class of aromatic organic arsenical compounds in which mercury was introduced.

It appeared to us particularly interesting to synthesize compounds where both mercury and arsenic were present in the molecule, as the combined effect of these elements, we thought, might be of greater therapeutic value. Only very few organic compounds containing mercury and arsenic have been described in the literature. Practically no data about their biological properties have been given.

We prepared mercurial derivatives of various phenylarsinic acids. Mercury could not be introduced into aromatic compounds containing trivalent arsenic, for instance, in arsphenamine, inasmuch as oxidation occurred almost immediately with the resulting formation of metallic mercury.

EXPERIMENTAL.

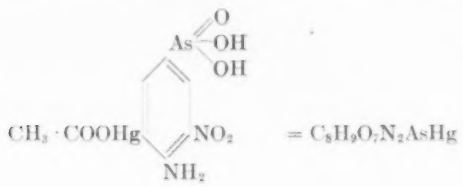
3-Nitroarsanilic Acid-Mercuric Acetate, No. 76.

10 gm. of 3-nitroarsanilic acid were suspended in about 75 cc. of water and dissolved by the addition of 22 cc. of 15 per cent sodium hydroxide (the amount necessary to form a disodium salt). This was mixed with a solution of 13.0 gm. of mercuric acetate dissolved in 70 cc. of water (12.1 gm. = 1 mol) containing a little acetic acid to prevent the formation of any basic salt. A yellow precipitate was thrown down immediately. The whole was allowed to stand for 48 hours in the cold with vigorous mixing from time to time. Then the precipitate was filtered off, washed thoroughly with water, then methyl alcohol, and finally with ether, and dried in the desiccator over sulfuric acid.

Yield = 15.3 gm. = 77 per cent of the theoretical.

	Nitrogen. *per cent*	Mercury. *per cent*	Arsenic. *per cent*
Analysis.—Calculated	5.38	38.46	14.42
Found	5.73	38.38	14.52

These results are in agreement with the following formula:



$= C_8H_9O_7N_2AsHg$

Properties.—A bright yellow powder soluble in very dilute sodium hydroxide on warming. It is slightly soluble in methyl alcohol, insoluble in cold or warm ethyl alcohol, ether, and acetone. Soluble in glacial acetic acid at ordinary temperature, also in 15 per cent acetic acid on warming and in 10 per cent hydrochloric acid.

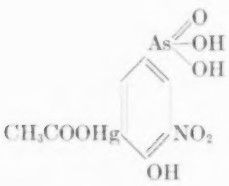
3-Nitro-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 95.

10 gm. of 3-nitro-4-hydroxyphenylarsinic acid were dissolved using 50 cc. of water and 20 cc. of 15 per cent sodium hydroxide. To the deep yellow solution were added 12.5 gm. of mercuric acetate (12 gm. = 1 mol) in 65 cc. of water and a little acetic acid. A yellow precipitate was formed which was insoluble in 0.5 per cent sodium hydroxide. The whole was heated on a water bath for 1½ to 2 hours when a sample dissolved completely in 0.5 per cent sodium hydroxide, thereby showing the absence of any free mercury ions. After thorough cooling, the precipitate was filtered off, washed thoroughly with water, methyl alcohol, and ether, and then dried in a desiccator over sulfuric acid.

It may be purified by dissolving in dilute sodium hydroxide and reprecipitating with acetic acid.

Yield = 13.5 gm. = 68 per cent of the theoretical.

	Nitrogen. *per cent*	Mercury. *per cent*	Arsenic. *per cent*
Analysis.—Calculated.....	2.69	38.39	14.40
Found.....	2.61 2.82	38.05	14.50



$= C_8H_8O_8N\ AsHg$

Properties.—A yellow powder soluble in dilute sodium hydroxide forming a yellow solution, insoluble in both cold and warm methyl and ethyl alcohol, ether, acetone, and glacial acetic acid.

3:5-Dinitro-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 126.

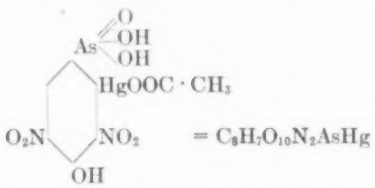
10 gm. of 3:5-dinitro-4-hydroxyphenylarsinic acid were dissolved in 10 cc. of methyl alcohol by warming on a water bath. 12 gm. of mercuric acetate (10.0 gm. = 1 mol) were dissolved in 75 cc. of methyl alcohol with the addition of a few drops of acetic acid, and the two solutions mixed in the cold. The mixture was warmed on the water bath with a reflux condenser for about 10 hours until no free mercury ions could be detected by 0.5 per cent sodium hydroxide.

The precipitate was filtered off, washed thoroughly with methyl alcohol and then with ether, and dried in the desiccator.

Yield = 11.0 gm. = 60 per cent of the theoretical.

	Nitrogen. *per cent*	Mercury. *per cent*	Arsenic. *per cent*
Analysis.—Calculated	4.96	35.34	13.25
Found	4.72	35.35	13.60

These results correspond with the following formula:



$= C_8H_7O_{10}N_2AsHg$

Properties.—A bright yellow powder only partially soluble in dilute sodium hydroxide. A pale yellow turbidity persists. It is insoluble in methyl and ethyl alcohol, ether, and acetone.

3-Amino-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 96.

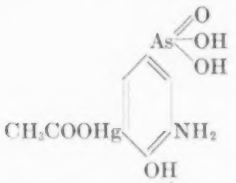
10 gm. of 3-amino-4-hydroxyphenylarsinic acid were dissolved in 75 cc. of water and 21 cc. of 15 per cent sodium hydroxide at room temperature (because warming an alkaline solution of the above amino compound would oxidize it). A dark brown solution was thus formed. This was mixed with a solution of 14.0 gm. of mercuric acetate (13.6 gm. = 1 mol) dissolved in 70 cc. of water and a little glacial acetic acid. The mixture was kept cooled in ice water.

A light brown precipitate formed immediately. The whole was well shaken for about ½ hour, during which time the color of the precipitate gradually darkened. It was filtered off, washed with water, methyl alcohol, and ether, and dried in the desiccator.

It may be purified by dissolving in dilute sodium hydroxide and reprecipitating with acetic acid.

Yield = 17.5 gm. = 83 per cent of the theoretical.

	Nitrogen. *per cent*	Mercury. *per cent*	Arsenic. *per cent*
Analysis.—Calculated.....	2.85	40.73	15.27
Found.....	2.83	40.60	15.25



$= C_8H_{10}O_5NAsHg$

Properties.—A brown powder soluble in dilute sodium hydroxide. This solution splits off metallic mercury within a few minutes which is deposited as a fine, gray powder. It is insoluble in the usual organic solvents, slightly soluble in cold glacial acetic acid and also 10 per cent hydrochloric acid.

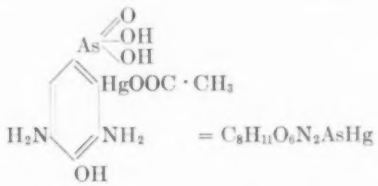
3:5-Diamino-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 125.

10 gm. of 3:5-diamino-4-hydroxyphenylarsinic acid were dissolved in 100 cc. of water and 22 cc. of 15 per cent sodium hydroxide, thereby forming a disodium salt. A brown solution was obtained. This was mixed with a solution of 13.5 gm. of mercuric acetate (13.0 gm. = 1 mol) dissolved in 70 cc. of water and a little acetic acid. The mixture was kept cold by immersing in ice water and was vigorously mixed for about ½ hour, at the end of which time a sample of the precipitate dissolved completely in 0.5 per cent sodium hydroxide. The precipitate was filtered off, washed thoroughly with water, methyl alcohol, and ether, and dried in the desiccator.

It may be purified by dissolving in 1 per cent sodium hydroxide and reprecipitating with dilute acetic acid.

Yield = 13 gm. = 65 per cent of the theoretical.

	Nitrogen. per cent	Mercury. per cent	Arsenic. per cent
Analysis.—Calculated	5.53	39.53	14.82
Found	6.06	38.87	14.84



$= C_8H_{11}O_6N_2AsHg$

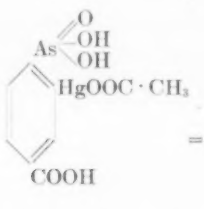
Properties.—A dark brown powder soluble in very dilute sodium hydroxide. The solution on standing splits off metallic mercury. Insoluble in the usual organic solvents. Partially soluble in cold glacial acetic acid. Soluble in 10 per cent hydrochloric acid.

4-Carboxyphenylarsinic Acid- or p-Benzarsinic Acid-Mercuric Acetate, No. 127.

10 gm. of *p*-benzarsinic acid were dissolved in 75 cc. of water and 11.0 cc. of 15 per cent sodium hydroxide by warming on the water bath. This was mixed at ordinary temperature with a solution of 13.5 gm. of mercuric acetate (13.0 gm. = 1 mol) in 70 cc. of water. A white precipitate formed immediately. The whole was warmed on the water bath for about 1 hour, when a sample of the filtrate no longer showed the presence of mercury ions. The precipitate was filtered off, washed thoroughly with water, methyl alcohol, and ether, and dried in the desiccator.

Yield = 13.5 gm. = 67 per cent of the theoretical.

	Mercury. per cent	Arsenic. per cent
Analysis.—Calculated	39.68	14.88
Found	39.80 39.75	15.13



$= C_9HgO_7AsHg$

Properties.—A cream-colored powder insoluble in sodium hydroxide, a yellow precipitate being formed. Soluble in dilute hydrochloric acid, concentrated sodium chloride solution, and in glacial acetic acid on warming. Insoluble in hot or cold methyl or ethyl alcohol, ether, and acetone.

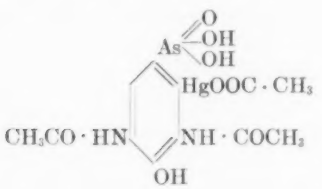
Diacetyl-3:5-Diamino-4-Hydroxyphenylarsinic Acid-Mercuric Acetate, No. 97.

10 gm. of diacetyl-3:5-diamino-4-hydroxyphenylarsinic acid were dissolved in 175 cc. of methyl alcohol by warming on the steam bath. The dark brown solution obtained was mixed in the cold with 10 gm. of mercuric acetate (9.6 gm. = 1 mol) dissolved in 60 cc. of methyl alcohol. After shaking for about 20 minutes a dark gray precipitate settled out, which was filtered off and washed thoroughly with methyl alcohol and ether, and dried in a desiccator.

This compound could not be further purified because of its instability. The results obtained for mercury are not quite in accord with the assumed formula. No other formula could be suggested. The nitrogen and arsenic values were found to agree with the assumed formula.

Yield = 15 gm. = 86 per cent of the theoretical.

	Nitrogen. *per cent*	Mercury. *per cent*	Arsenic. *per cent*
Analysis.—Calculated.....	4.75	33.90	12.71
Found.....	4.65	36.40	13.05



$= C_{12}H_{15}O_8N_2AsHg$

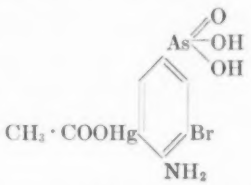
Properties.—A gray powder soluble in dilute sodium hydroxide forming a brown solution. This on standing becomes turbid, and finally metallic mercury is split off. It is insoluble in the usual organic solvents. Partly soluble in cold glacial acetic acid.

3-Bromoarsanilic Acid-Mercuric Acetate, No. 121.

10 gm. of 3-bromoarsanilic acid were dissolved in 100 cc. of water. To this were added 18 cc. of sodium hydroxide and a solution of 11.0 gm. of mercuric acetate (10.5 gm. = 1 mol) in 65 cc. of water and the whole was well mixed. After heating on a steam bath for about 3 hours, no mercury ions could be detected by 0.5 per cent sodium hydroxide. After cooling, the precipitate was filtered, washed with water, methyl alcohol, and ether, and then dried in the desiccator.

Yield = 15 gm. = 80 per cent of the theoretical.

	Nitrogen. per cent	Mercury. per cent	Arsenic. per cent
Analysis.—Calculated	2.53	36.10	13.54
Found	2.74	36.15	13.52



$= C_8H_9O_5NBrAsHg$

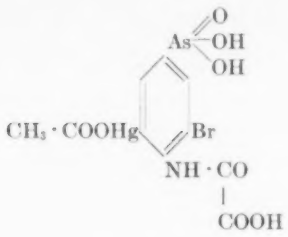
Properties.—A white powder insoluble in the usual organic solvents. Soluble in dilute sodium hydroxide, warm glacial acetic acid, and 10 per cent hydrochloric acid.

3-Bromooxalylarsanilic Acid-Mercuric Acetate, No. 122.

10 gm. of 3-bromooxalylarsanilic acid were dissolved in 150 cc. of water and 15 cc. of sodium hydroxide, to which was added a solution of 9.5 gm. of mercuric acetate (8.6 gm. = 1 mol) dissolved in 60 cc. of water. The mixture was heated on the steam bath for about 2 hours when a sample dissolved completely in sodium hydroxide. The precipitate was filtered, washed with water, ethyl alcohol, and ether, and dried in the desiccator.

Yield = 8.5 gm. = 50 per cent of the theoretical.

	Nitrogen. per cent	Mercury. per cent	Arsenic. per cent
Analysis.—Calculated	2.24	31.95	11.98
Found	2.19	32.30 32.37	11.65



$= C_{10}H_9O_5NBrAsHg$

Properties.—A white powder insoluble in organic solvents and acetone. Soluble in very dilute sodium hydroxide. If this solution is allowed to stand metallic mercury splits off.

Biological Experiments.

These new compounds were subject to a series of routine biological tests for (*a*) antiseptic and germicidal activity for staphylococci, *Bacillus typhosus*, and a spore-bearing microorganism *Bacillus anthracis*, (*b*) toxicity for white rats, and (*c*) for trypanocidal activity against infections with *Trypanosoma equiperdum* in white rats.

Results of Antiseptic or Bacteriostatic Tests.—In conducting these tests varying dilutions of solutions of the different compounds in amounts of 1 cc. were placed in sterile test-tubes and further diluted by the addition of 5 cc. of nutrient broth and seeding with 0.5 cc. of filtered, 24 hour broth cultures of the respective microorganisms. Each series of tubes was observed over a period of 5 days and the highest final dilution showing retardation of growth was recorded as the antiseptic or bacteriostatic strength of the compound. Subcultures or solid media have regularly shown that these antiseptic values do not correspond to the germicidal activity inasmuch as viable microorganisms may be recovered, but the technique is very simple, serves to bring out the finer differences in antiseptic values of the different compounds, and has proved of much value in former studies with compounds of this class for determining their influence upon bacterial activity.

The results of tests with this series of new compounds and with mercuric chloride and mercurophen are summarized in Table I and have shown the following.

1. As a general rule a higher antiseptic activity was displayed against staphylococci than *Bacillus typhosus*. This is particularly true of sodium oxy-mercury orthonitrophenolate (mercurophen) as previously described (7) and is apparent with several of the new compounds included in this series; namely, Nos. 76, 95, 97, 121, and 122. Compounds 96 and 125 did not show this peculiar and interesting superior antiseptic activity for staphylococci.

2. Two of these compounds, namely Nos. 97 and 121, have also displayed striking antiseptic activity for the bacilli and spores of anthrax, which is a distinguishing characteristic of mercurophen.

TABLE I.

Results of Antiseptic or Bacteriostatic Tests of Compounds in a Menstruum of Nutrient Broth.

Substance.	Mercury.	Highest antiseptic solution for		
		Staphylococcus aureus.	*Bacillus typhosus.*	*Bacillus anthracis.*
	per cent			
Bichloride Hg.....	74	1:600,000	1:360,000	1:600,000
Mercurophen.....	53	1:2,400,000	1:360,000	>1:2,400,000
No. 76.....	38.3	1:420,000	1:180,000	1:180,000
" 95.....	38	1:180,000	1:42,000	1:36,000
" 96.....	40.6	1:60,000 (?)	1:60,000	1:54,000
" 97.....	36.4	1:420,000	1:180,000	>1:2,000,000
" 121.....	36.1	1:480,000	1:180,000	1:600,000
" 122.....	32.3	1:540,000	1:180,000	1:180,000
" 125.....	38.8	1:360,000	1:240,000	1:120,000

Additional tests with compounds of this series which could be prepared in sufficiently concentrated solutions, have shown that a marked reduction in antiseptic activity occurs in the presence of large amounts of protein, as when the tests are conducted in a menstruum of serum. The results of tests shown in Table II were obtained by adding to varying dilutions of the compounds in water in amounts of 1 cc. an equal amount of sterile human serum and seeding with 0.05 cc. of filtered, 24 hour broth cultures of *Staphylococcus aureus;* the results indicate the highest antiseptic or bacteriostatic activities of the various compounds in a menstruum of 50 per cent human serum.

TABLE II.

Results of Antiseptic or Bacteriostatic Tests of Compounds in a Menstruum of Human Serum for Staphylococcus aureus.

Substance.	Mercury.	Highest antiseptic solution for *Staphylococcus aureus*.
	per cent	
Bichloride Hg	74	1:5,400
Mercurophen	53	1:6,000
No. 76	38.3	1:6,000
" 96	40.6	1:4,800
" 121	36.1	1:6,000
" 123	32.3	1:4,800
" 125	38.8	1:5,400

Results of Toxicity Tests.—These tests were conducted by injecting white rats intravenously with varying doses of the new compounds per kilo of body weight and so prepared that the dose for each animal was contained in exactly 1 cc. The injections were made in a saphenous vein and at the rate of 1 cc. per minute. Following injection the animals were observed for a period of 2 weeks. The results of these tests are shown in Table III and summarized in Table IV with the inclusion of mercuric chloride and mercurophen for purposes of comparison.

A few compounds were also tested by intramuscular injection into the muscles of the thigh of white rats and the results with Nos. 96 and 97 are shown in Table V.

Previous studies in these laboratories with ordinary mercurial compounds (8) have shown that toxicity is somewhat proportional to the content in mercury and this condition is apparently true of these new compounds as judged by the highest tolerated doses at the end of a 10 day period of observation. An exception to this general rule is apparent with mercurophen which is generally borne by experimental animals in doses higher than expected according to the percentage of mercury present and to which attention has been drawn in the investigations previously mentioned (7).

Results of Trypanocidal Tests.—These experiments were conducted by infecting white rats with approximately known numbers (9) of a virulent strain of *Trypanosoma equiperdum* by intraperitoneal or subcutaneous injection 24 hours before the intravenous injection of the compounds in varying dosage per kilo of

TABLE III.

Results of Toxicity Tests by Intravenous Injection in Rats.

Compound No.	Name of compound.	Mercury.	Weight of rat.	Dose per kilo	Results in days.												
					1	2	3	4	5	6	7	8	9	10	11	12	13	14
		per cent	*gm.*	*mg.*														
95	3-Nitro-4-hydroxy-phenylarsinic acid-mercuric acetate.	38.05	79	10	*D													
			117	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			78	4	–	–	D											
			180	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
96	3-Amino-4-hydroxy-phenylarsinic acid-mercuric acetate.	40.6	160	30	D													
			195	20	D													
			130	10	–	–	–	D										
			185	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
97	Diacetyl-3: 5-di-amino-4-hydroxy-phenylarsinic acid-mercuric acetate.	36.4	107	10	–	–	–	–	D									
			98	8	–	–	–	D										
			138	6	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			114	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
121	3-Bromoarsanilic acid-mercuric acetate.	36.15	140	30	D													
			350	20	D													
			120	10	–	–	–	D										
			185	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
122	3-Bromooxalyl-arsanilic acid-mercuric acetate.	32.3	120	30	D													
			130	20	D													
			160	10	–	D												
			115	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
125	3: 5-Diamino-4-hydroxyphenyl-arsinic acid-mercuric acetate.	38.87	165	30	–	–	D											
			155	20	D													
			100	10	–	–	–	D										
			130	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–

* Died.

body weight; each dose was prepared separately and contained in 1 cc. for each animal. Numerous controls were infected at the same time and in the same manner but were not injected with the compounds. Following the injections of drugs the blood of

TABLE IV.

Summary of Toxicity Tests by Intravenous Injection in Rats.

Compound No.	Name of compound.	Mercury.	Highest tolerated dose per kilo.	
			2 days.	10 days.
		per cent	*mg.*	*mg.*
95	3-Nitro-4-hydroxyphenylarsinic acid-mercuric acetate.	38.05	8	2-8
96	3-Amino-4-hydroxyphenylarsinic acid-mercuric acetate.	40.6	10	5
97	Diacetyl-3:5-diamino-4-hydroxyphenylarsinic acid-mercuric acetate.	36.4	10	6
121	3-Bromoarsanilic acid-mercuric acetate.	36.15	10	
122	3-Bromooxalylarsanilic acid-mercuric acetate.	32.3	5	5
125	3:5-Diamino-4-hydroxyphenylarsinic acid-mercuric acetate.	38.87	10	5
0	Mercuric chloride.	74	6	3-4
0	Mercurophen.	53	10	8

TABLE V.

Results of Toxicity Tests by Intramuscular Injection in Rats.

Compound No.	Name of compound.	Weight of rat.	Dose per kilo.	Results in days.												
				1	2	3	4	5	6	7	8	9	10	11	12	13	14
		gm.	*mg.*														
96	3-Amino-4-hydroxyphenylarsinic acid-mercuric acetate.	74	41	–	–	–	–	–	–	–	–	–	–	D			
		179	14	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		130	13	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		89	13	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		124	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
97	Diacetyl-3:5-diamino-4-hydroxyphenylarsinic acid-mercuric acetate.	134	24	–	–	–	–	–	–	–	–	–	–	–	–	–	–
		100	22	–	–	–	–	–	–	–	D						
		130	14	–	–	–	–	–	–	–	–	D					
		175	9	–	–	–	–	–	–	–	–	–	D				
		69	7	–	–	–	–	–	–	–	–	–	–	–	–	–	–

each animal was examined daily for trypanosomes by direct microscopical examination until death occurred.

The results of these therapeutic tests are shown in Tables VI and VII. As previous studies have shown (10) mercurial compounds cannot be given in sufficient dosage to exert more than a minor and temporary influence upon experimental trypanosomiasis with this strain of trypanosomes and similar results were observed with the new compounds herein described.

TABLE VI.

*Results of Therapeutic Tests with Trypanosoma equiperdum.**

Compound. No.	Name of compound.	No.	Weight of rat.	Dose per kilo.	Results in days.						
					1	2	3	4	5	6	7
			gm.	*mg.*							
95	3-Nitro-4-hydroxyphenyl-arsinic acid-mercuric-acetate.	1	90	12	−	+	+	+	+	+	D
		2	87	10	−	+	+	+	+	+	D
		3	84	8	D						
		4	85	6	−	+	+	+	+	+	D
96	3-Amino-4-hydroxyphenylar-sinic acid-mercuric acetate.	5	145	8	−	+	+	D			
		6	220	4	−	+	+	+	D		
		7	210	2	−	+	+	+	D		
Controls.	0	8	115	0	Few.	+	+	+	+	D	
		9	144	0	"	+	+	+	+	+	D
		10	114	0	"	+	+	+	+	D	
		11	140	0	"	+	+	+	+	D	

* Rats infected by intraperitoneal injection of 180,000 trypanosomes 24 hours before the intravenous injection of the drugs.

Entirely different results may be expected in tests with rabbits infected with *Treponema pallidum* and we hope to be able to report later on this phase of the problem, when these laborious experiments are sufficiently concluded to permit of drawing comparisons and conclusions.

Further tests conducted with a new technique previously described (11), consisting in mixing in test-tubes the blood of rats showing the presence of very large numbers of these trypanosomes with an equal quantity of solutions of the new compounds

and testing for trypanocidal activity by injecting portions of the mixture intraperitoneally in rats after standing for varying periods of time at 37°C. in a water bath, have shown a high degree of trypanocidal activity on the part of several of these compounds; the results observed with two of them (Nos. 95 and 97) are shown in Table VIII.

TABLE VII.

*Results of Therapeutic Tests with Trypanosoma equiperdum.**

Compound No.	Name of compound.	Weight of rat.	Dose per kilo.	Results in days.				
				1	2	3	4	5
		gm.	*mg.*					
95	3-Nitro-4-hydroxyphenylarsinic acid-mercuric acetate.	185	2	Few.	+	+	+	D
		145	1	"	+	+	D	
		140	½	"	+	D		
97	Diacetyl-3: 5-diamino-4-hydroxyphenylarsinic acid-mercuric acetate.	180	2	"	+	+	D	
		175	1	"	+	D		
		175	½	"	+	+	D	
121	3-Bromoarsanilic acid-mercuric acetate.	220	2	"	+	+	D	
		190	1	"	+	+	D	
		185	½	"	+	+	D	
122	2-Bromooxalylarsanilic acid-mercuric acetate.	160	2	"	+	D		
		200	1	"	+	+	D	
		210	½	"	+	+	D	
Controls.		170	0	"	+	+	+	D
		160	0	+	+	D		
		175	0	+	+	+	D	

* Rats infected by subcutaneous injection of 800,000 trypanosomes 24 hours before the intravenous injection of the drugs.

The technique is quite delicate and serves to show whether or not compounds too toxic for administration in sufficient amounts to influence experimental trypanosomiasis in living animals possess trypanocidal activity as tested by this combined *in vitro-vivo* method.

TABLE VIII.

Results of Trypanocidal Tests with the Combined in Vitro-Vivo Technique.

Compound No.	Name of compound.	Weight of rat.	Dose.*	Results in days.									
				1	2	3	4	5	6	7	8	9	10	15
		gm.	*mg.*											
95	3-Nitro-4-hydroxy-	84	0.001	–	–	–	–	D						
	phenylarsinic acid-	77	0.0005	–	–	–	–	–	D					
	mercuric acetate.	55	0.00025	–	–	–	–	D						
		114	0.000125	–	–	–	–	D						
		63	0.000625	–	–	–	–	–	–	–	–	–	–	–
97	Diacetyl-3:5-di-	81	0.001	–	–	–	–	–	–	–	–	–	–	–
	amino-4-hydroxy-	122	0.0005	–	–	–	–	–	–	–	–	–	–	–
	phenylarsinic	82	0.00025	–	–	–	–	–	–	–	–	–	–	–
	acid-mercuric	102	0.000125	–	–	–	–	–	–	–	–	–	–	–
	acetate.	92	0.0000625	–	–	–	–	+	+	+	D			
	0	65	0	+	+	+	+	+	D					
Controls.		62	0	–	+	+	+	+	D					
		60	0	–	+	+	+	+	D					

* Final amount of drug acting upon the trypanosomes contained in the blood of rats.

Conclusions.

1. The new compounds herein described possess less antibacterial activity than mercuric chloride but are likewise lower in content of mercury; a number of these compounds have shown a particularly increased activity against staphylococci and a few against a spore-forming bacillus, namely *Bacillus subtilis*, as compared with mercuric chloride.

2. The antibacterial activities of the new compounds tested showed a marked reduction in a menstruum rich in serum proteins.

3. The toxicity of these compounds for white rats bears a general relation to their content in mercury.

4. The new compounds possess trypanocidal activity as tested by an *in vitro-vivo* method but cannot be administered to living animals in sufficient amounts to appreciably influence the course of experimental trypanosomiasis.

Methods of Analysis.

Nitrogen was determined by the ordinary Kjeldahl method. In the cases of compounds containing nitro groups the latter were first reduced. Mercury and arsenic were determined from the same sample. 0.2 gm. was decomposed by 25 cc. of concentrated sulfuric acid, diluted with water, and the mercury and arsenic were precipitated together by hydrogen sulfide. After filtering and washing, the arsenic sulfide was dissolved in ammonia and the arsenic determined gravimetrically in the usual way as magnesium pyroarsenate. The mercuric sulfide was washed successively with methyl alcohol, carbon bisulfide, and acetone, dried, and weighed. The washing with organic solvents, etc. was repeated until the weight of the mercuric sulfide remained constant.[1]

Stability.

In order to determine the degree of firmness with which the mercury was attached to the nuclear carbon, we devised the following method. 100 mg. of the compound are dissolved in a small quantity of dilute sodium hydroxide in a 25 cc. glass-stoppered cylinder and dilute acetic acid added drop by drop until the precipitate, which forms, just barely redissolves. The solution is diluted with water up to 25 cc., 5 cc. of 5 per cent neutral ammonium sulfide are introduced, and the mixture is shaken. The time required for the complete precipitation of mercuric sulfide is noted. If this precipitate does not come down within 30 minutes the compound is regarded as being unaffected. The mixture is then heated to 80°C. and maintained at this temperature, noting the time of the precipitation of mercury. If the result is negative after ½ hour, a new solution of the compound is made up and treated this time with hydrogen sulfide, first at ordinary temperature, then at 80°C., and finally at its boiling temperature if necessary. None of the compounds described in this article required treatment with hydrogen sulfide. The results obtained are given in Table IX.

[1] This method of analysis was suggested to us by Dr. W. Jacobs and Dr. M. Heidelberger for which we take this occasion to express our appreciation.

TABLE IX.

Compound.	Ammonium sulfide in cold.	Ammonium sulfide at 80°C.
3-Nitroarsanilic acid-mercuric acetate.	Precipitated immediately.	
3-Nitro-4-hydroxyphenylarsinic acid-mercuric acetate.	Precipitated in 30 min.	
3:5-Dinitro-4-hydroxyphenylarsinic acid-mercuric acetate.	Precipitated immediately.	
3-Amino-4-hydroxyphenylarsinic acid-mercuric acetate.	Partial precipitation.	Complete after 19 min.
3:5-Diamino-4-hydroxyphenylarsinic acid-mercuric acetate.	Slight precipitate.	Complete precipitation in ½ hr.
Diacetyl-3:5-diamino-4-hydroxyphenylarsinic acid-mercuric acetate.	Precipitated immediately.	
p-Benzarsenic acid-mercuric acetate.	" "	
3-Bromoarsanilic acid-mercuric acetate.	" "	
3-Bromooxalylarsanilic acid-mercuric acetate.	" "	

SUMMARY.

1. The presence of the arsenic acid group in the molecule of organic compounds described in this paper apparently does not interfere with the entrance of the mercury group. The half complex compounds thus formed are characterized by a comparative stability in alkaline solution. The splitting off of metallic mercury occurs only in compounds containing an animo group which has also been frequently observed in non-arsenical compounds.

2. The process of formation of the organic arsenical mercury compounds and their chemical properties do not differ substantially from organic mercury compounds containing no arsenic acid group.

3. The toxic effect on the animal body as it appeared to us is mainly caused by the mercury group. The arsenic acid group neither increases nor decreases the toxicity of the compounds.

4. To our disappointment, the curative influence of these new compounds in experimental trypanosomiasis and also the germicidal effect *in vitro* were not superior to the ordinary organic mercury compounds.

5. As in every other chemotherapeutic investigation the apparently negative therapeutic results with the compounds herein described cannot be accepted as definitely characteristic for the arsenical mercury compounds. Our studies were limited to a comparatively small number of compounds and further studies might result in the discovery of a powerful trypanocidal or germicidal containing both mercury and arsenic.

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